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Monitoring the Genetic Health of Humans Accidentally Exposed to Ionizing Radiation of Cesium-137 in Goiânia (Brazil)

by

Aparecido Divino da Cruz
B.Sc., Universidade Católica de Goiás, 1989

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

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ABSTRACT

This thesis describes a long-term study in which the genetic health of a population accidentally exposed to ionizing radiation of cesium-137. The Goiânia (Brazil) radiological accident of September 1987 involved 249 individuals exposed to doses up to 7 Gy, and included four fatalities.

We have investigated the genetic effects of radiation exposure in this population using both cytogenetic and molecular endpoints in T-lymphocytes. The micronucleus assay differentiated between groups exposed to different levels of ionizing radiation. At the molecular level two methods were employed: 1) the *hprt* clonal assay; and 2) the determination of microsatellite instability. The *hprt* assay involves *in vitro* culturing of T-cells and the selection of 6-thioguanine-resistant *hprt* mutant clones which were then characterized at the molecular level using both RT-PCR and genomic analysis. Exposure to ionizing radiation initially elevated the mutation frequency but this effect gradually diminished, so that 4.5 years no significant increase was observed. This limitation makes the *hprt* T-cell assay unsuitable for the study of long term past exposure. Analysis of the spectrum of *hprt* mutations recovered from 10 individuals exposed to relatively high doses of ionizing radiation revealed a significant increase (3.8-fold) in the frequency of A:T → G:C mutations in the exposed group. This increase in A:T → G:C transitions is consistent with the effects of ionizing radiation in prokaryotes and lower eukaryotes and likely reflects the mispairing of radiation-induced thymine glycol with guanine. In addition, a two-fold

increase in the frequency of deletions not readily explained by slippage events and hence which may reflect ionizing radiation-induced DNA strand breakage was also observed.

Microsatellite instability was also investigated. Fluorescent PCR and automated DNA sequencer analysis, using genomic DNA from mononuclear cells, were used to investigate the frequency of microsatellite alterations in exposed and non-exposed populations. We examined a total of 200 and 190 alleles respectively and found that the microsatellite instability distribution in the two groups were not different. Our assay lacked sufficient sensitivity to discriminate between spontaneous and induced microsatellite instability and it is, therefore, not suitable for population monitoring.

Finally, despite the minimal database, we used the micronucleus and *hprt* mutant frequency data to estimate the risk associated with radiation exposure for the Goiânia population. The estimated genetic risk for the exposed group was approximately a 24-fold increase in dominant disorders in the first post-exposure generation. Moreover, the risk of carcinogenesis in this population was estimated to be increased by a factor in the range of 1.4 to 1.5 compared to the population at large.

Examiners:

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List of Abbreviations

6TG	6-Thioguanine
AB	A-bomb survivors
ALF	Automated laser fluorescent
APC	Antigen presenting cell
bp	Base pair
BSA	Bovine serum albumin
BSS	International basic safety standards for protection against ionizing radiation sources
CDA	Canonical discriminant analysis
cDNA	Complementary DNA
CE	Cloning efficiency
CEH	Center for Environmental Health
CI	Confidence interval
CV	Canonical variate
DD	Doubling dose
ddNTP	Dideoxy nucleotide triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide trisphosphate
DTT	Dithiotriol
FISH	Fluorescent *in situ* hybridization
FunLeide	Fundação Leide das Neves Ferreira
GMP	Guanosine monophosphate
HD	High dose
hprt (HPRT)	Hypoxanthine-guanine phosphoribosyltransferase
IGR	Instituto Goiano de Radioterapia
IL-2	Inter-leukin-2
IMP	Inosine monophosphate
Inr	Initiator
LD	Low dose
LET	Linear energy transfer
LN_2	Liquid nitrogen
lnMF	Natural logarithm of mutant frequency
MANOVA	Multiple analysis of variance
MC	Mutational component
MF	Mutant frequency
MF/PE	Corrected mutant frequency
MHC	Major histocompatibility complex
MIN	Microsatellite instability
Mo-MLV	Moloney murine leukemia virus
MN	Micronucleus
MNC	Mononuclear cells
mPCR	Multiplex PCR
mRNA	Messenger ribonucleic acid
p	Chromosome's short arm
PCR	Polymerase chain reaction
PE	Plating efficiency
PHA	Phytohaemagglutinin
PRPP	5-Phosphoribose1-pyrophosphate
q	Chromosome's long arm
RNA	Ribonucleic acid
RT	Reverse transcriptase
SD	Standard deviation
SE	Standard error
SS	Spontaneous spectrum
SSD	Sanitary Surveillance Division
SV40	Simian virus 40
TCR	T-cell receptor
TFIID	Transcription factor IID
TG^R	Thioguanine-resistant
TMAC	Tetramethylammonium chloride
UCG	Universidade Católica de Goiás
vF	Variant frequency

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Acknowledgments

"The man of science appears to be the only person who has something to say just now, and the only man who does not know how to say it."

- Sir James Barrie

First I wish to thank all my family for their support, encouragement, and prayers. Sincere gratitude to my mother, Pedro, Damiana, Marcelo, Glênio, Raphael, Alex, Bernadete, and my cousins Cida, Lúcia, Admirson, and Oendis, and my aunt Geni.

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Dedications

This thesis is dedicated to

My mother, **Aparecida**, *and to the memory of my father,* **João**, *for always encouraging my dreams and teaching me to follow my heart, no matter what.*
My big brother **Pedro** *for all his support and wonderful friendship.*
Damiana *and* **Marcelo**, *my God's greatest gifts, for trusting me with their unconditional love.*
My dear friend **Sean** *for giving me a family and so much care while abroad.*

"Perhaps the greatest social service that can be rendered by anybody to the country and to mankind is to bring up a family."

- George Bernard Shaw

"Ela era feia. Mas quando apaguei as luzes do ferro-velho e notei que ela brilhava, me apaixonei. Nunca pensei que aquela pedra maravilhosa fosse fazer isso comigo."

- Devair Alves Ferreira
in: Ciência Hoje (Suppl.7) 40:44

"It was ugly. But, when I turned off the lights of the junk yard and noticed that it glowed, I was bewitched. I never thought that such an extraordinary stone would do this to me."

Translated by the author

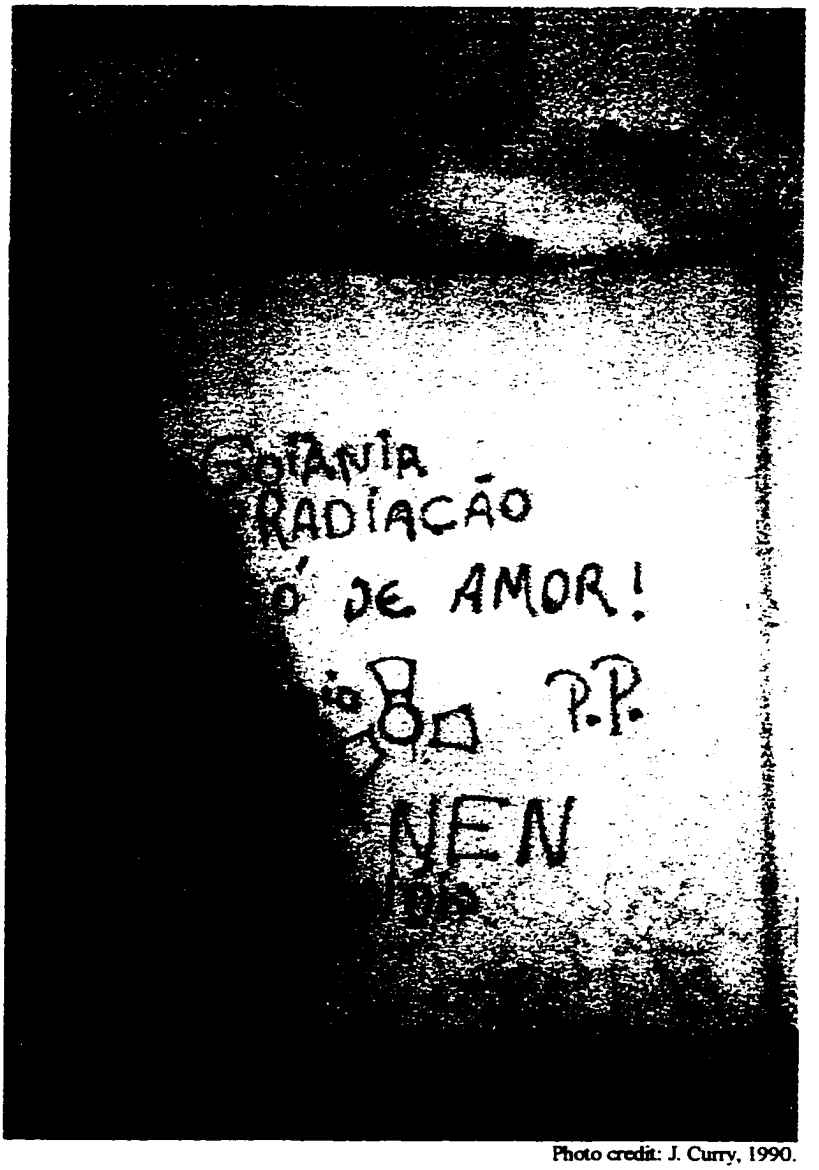

Photo credit: J. Curry, 1990.

"Goiânia, irradiation only of love! Like us."

Layperson's Introduction

"There is no concept so difficult that it can't be explained in a simple way."
- Albert Einstein

It is suspected that many human diseases are caused by chemicals, radiation, and other agents which can be found in the environment. Many of these agents have been studied in an attempt to understand how they contribute to human illness, mainly cancer. In the case of radiation, excessive amounts of energy hit the cell, damaging DNA and protein molecules. Because of this, cells can either be killed or modified (mutated) which leads to impaired cellular function.

Unfortunately, a serious radiological accident occurred in Goiânia (Brazil) in 1987. During the accident, 249 people were exposed to various levels of ionizing radiation and four fatalities resulted. The severity of radiation exposure depended on the proximity and the length of time individuals were exposed to the source. Several of the accident victims volunteered to donate blood samples annually, over a period of six years, providing a unique opportunity to study the nature of DNA mutations in a recently exposed human population.

Improved scientific methods have been used to investigate the biological effects of radiation exposure in living organisms, including humans. By using these techniques we can ask two questions. How does radiation damage the DNA? Is there an increased risk for cancers associated with exposure to radiation? Some of the tests we used included:

1. The T-cell clonal assay which used cells taken from the blood sample and allowed only DNA damaged cells to grow in a test tube;

2. Polymerase chain reaction (PCR), a technique that allowed the copying of the damaged DNA many times. This step is necessary to generate enough DNA molecules to be studied;
3. Automated DNA sequencing - with the aid of a computer the genetic coding, or the chemical components, of the copied DNA fragments (protein building instructions) were analyzed. The nature of the DNA from the exposed people were then compared to the DNA of unexposed individuals, called the control group.

This dissertation will discuss the follow-up study on the Goiânia population from its beginning in 1990. The first chapter describes previous scientific knowledge about the accident itself, human radiation exposure, the specific gene and the cells we studied (the *hprt* gene and T-lymphocytes). Chapter II discusses the relationship between damaged DNA and exposure to radiation, including the association with age and smoking habits. In chapter III we looked at the same individuals over three subsequent samplings to see if the type and frequency of the DNA damage would remain the same. We found that the higher the exposure, the higher the frequency of DNA damage. However, over time there was a gradual decrease in the frequency of damage. After 4.5 years, the level of damage in the exposed people showed no difference from the unexposed ones. Chapter IV compares the type of DNA damage found in the exposed individuals from the Goiânia population to other similar studies, including a study on A-bomb survivors. The main finding was that the genetic coding of the DNA was altered in different ways. The main changes affected sites labeled A:T. These sites were damaged 4 times more often in the exposed population, than in the unexposed population. Furthermore, the exposed individuals were two times more likely to have deletions of DNA fragments within the gene we studied. This means

that there was a cellular loss of DNA molecules, and consequently, a loss of building instruction for that cell and its progeny. Both changes and losses of DNA content increase the inability of the cell to reproduce itself accurately which is one of the major steps toward cancers. Another test used for checking alterations to the DNA is the microsatellite instability assay as described in chapter V. The microsatellite instability assay looks at areas of DNA molecules that normally contain repetitive sequences, namely microsatellites. Any changes, either additions or deletions, to the repetitive sequences are considered microsatellite instabilities. Utilizing this assay we did not find statistically significant differences between the exposed and non-exposed individuals. Finally, in chapter VI we examined the possible genetic harm and the risk of cancer development for the discrete Goiânia population. The risk of being born with a dominant genetic disorder translates into less than one child of the first generation of the exposed individuals. With respect to the risk of cancer development, the exposed population was found to be at 1.5 time higher risk than the Goiânia population at large.

Our general conclusions were that there was a decrease in mutant frequencies over time, a small increased risk of developing cancer, and an almost irrelevant genetic risk for the next generation.

Introduction and Thesis Rationale

The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact."
- T.H. Huxley

Environmental mutagens and carcinogens have been suspected as the cause of many human diseases. To date, a number of short-term approaches utilizing both eukaryote and prokaryote models are used to identify potential mutagens and carcinogens as well as to assess their impact on the environment. The results obtained in such studies are used to eliminate such agents from the environment or to develop strategies to minimize inevitable exposures.

Over the last five decades, intensive study has been conducted to understand the biological effects of exposure to ionizing radiation in humans at the molecular, cellular, and organismic levels. Despite these efforts, little is known about the mechanisms responsible, perhaps because of the complex nature of ionizing radiation and its complex interactions with biological matter. Nevertheless, exposure to ionizing radiation has been established as a hazard to human health by contributing to the mutational load and increasing the cancer incidence in exposed populations. This knowledge has clearly caused widespread concern over the biological effects of radiation exposure.

As biotechnological approaches have become available, they have allowed more detailed studies and a better understanding of the biological effects of exposure to ionizing radiation. In particular, it is now possible to determine the nature of mutations in an individual at the DNA sequence level. Such technological investigation allows for a more precise interpretation of the molecular events underling radiation-induced mutations and contributes to defining the mutational specificity of ionizing radiation.

On September 13th, 1987 in Goiânia (Brazil), a chain of unfortunate events led to a serious radiological accident that became known in the scientific community as simply the "Goiânia accident." During the two weeks following the accident, 249 people received substantial exposure to ionizing radiation of ^{137}Cs, resulting in four fatalities. Individual dose estimates ranged from near zero up to 7 Gy. In addition, personnel taxed with the subsequent patient care and cleanup were also exposed to low doses of ionizing radiation. This unfortunate accident, however, has provided a unique opportunity to study the nature of ionizing radiation-induced mutations in humans. Moreover, the actions undertaken by the Brazilian authorities following the accident resulted in an exposed group which was ideal for a follow-up study. The considerable efforts made to determine individual exposures, the continuing cytogenetic follow-up, and the detailed monitoring of the clinical health of the exposed individuals, maximize the potential value of our study.

Following the accident, the institute "Fundação Leide das Neves Ferreira (FunLeide)" was created by the Governor of the State of Goiás. This institute has the mandate of studying the long-term consequences of the Goiânia accident, including its medical, psychological, epidemiological, cytogenetic, and social aspects. In 1989, a mutual agreement between the FunLeide, the Universidade Católica de Goiás (UCG), and a Canadian institute, the Center for Environmental Health (CEH), was established. In this agreement, the CEH was granted access to the patients and their blood, on an annual basis, in order to carry out a follow-up monitoring study of the consequences of radiation exposure in that population.

The overall proposal of the follow-up study was the monitoring of the genetic health of the individuals exposed to ionizing radiation during the Goiânia accident. The

study involved the application of biotechnology to investigate the mutagenic effects of ionizing radiation at both cytogenetic and molecular levels, as well as to determine the consequences of radiation to the exposed and general populations by estimating the risk of carcinogenesis and genetic harm associated with radiation exposure. The applied strategy involved the use of methodologies and a combination of several techniques, such as the growth of T-lymphocytes under selective and non-selective conditions, polymerase chain reaction (PCR), automated DNA sequencing, and non-sequencing applications of an automated DNA sequencer, to name just a few.

The general objective of the long-term study was to determine the DNA damage caused by ionizing radiation, as well as to investigate the nature of mutation in the exposed population in Goiânia. The blood samples were collected at yearly intervals from both exposed and control populations of Goiânians, on a voluntary basis. The control group consisted of unexposed individuals selected from unexposed neighbours, family members, and the FunLeide's workers. Although this accident provides a rare opportunity to investigate the radiation-induced mutations in people, *in vivo*, it lacks the refinement of experimental studies conducted under the lens of previously designed protocols. In addition, accidental exposures generally involve complex populations displaying the normal human heterogeneity which impose some limitations. This is especially true, considering both the limited number of individuals available and the availability of the samples. We must, therefore, emphasize that some of these limitations are unavoidable and hence were present during the development of this study. The inherent limitations are addressed accordingly, when appropriate, throughout this dissertation.

This dissertation will deal with the outcome of the follow-up study on the Goiânia population which began in 1990 and which was conducted at both the FunLeide and the CEH. Chapter I sets the background for this study and includes discussions on the Goiânia radiological accident and individual dose estimations for the exposed population; the nature of ionizing radiation and its biological effects; the *hprt* gene and the selection assay; and finally T-lymphocyte development, cell cycle, and receptors as they pertain to this study. Chapter II describes and discusses the relationship between micronucleus frequency and exposure to ionizing radiation, including correlation with age and smoking habits as possible confounding factors affecting micronucleus frequency. Chapter III discusses the mutation frequency over time in a longitudinal study of three cohorts of individuals exposed to high levels of ionizing radiation. Chapter IV discusses the nature of radiation-induced mutations found in 10 individuals exposed to high doses of ionizing radiation. Chapter IV also compares our findings with a low dose-exposed cohort from the Goiânia population and to a group of A-bomb survivors. In addition, we further compare our results to the background mutations from unexposed individuals. The latter group includes data from the *hprt* database (Cariello *et al.*, 1994), a control group for the A-bomb survivors, and a Brazilian control group. Chapter V discusses the potential use of the frequency of microsatellite instability as indicators of somatic damage induced by ionizing radiation which is closely associated with the development of malignancies. Finally, chapter VI discusses the risk associated with radiation exposure in Goiânia; both the risk of cancer and genetic harm are considered.

CHAPTER I - Background

1. The Goiânia Radiological Accident

1.1. The Accident and Its Implications

Goiânia is located on the central Brazilian plateau and is the capital of Goiás State. The area is well known for its cereal farms and cattle ranches which are major contributors to the region's economy. Goiânia is a major city with a cosmopolitan population of about one million. It is a modern, well developed city, with access to modern medical and industrial technologies.

The community was serviced by the Instituto Goiano de Radioterapia (IGR), a private radiotherapy clinic which was relocated in late 1985. In the process of relocating the IGR, a ^{137}Cs radiotherapy unit was unfortunately abandoned and despite the legal requirement, the licensing governmental office was not properly notified. Shortly following the relocation, the premises of the former radiotherapy institute was partially demolished, leaving the ^{137}Cs radiotherapy unit in an extremely vulnerable situation (Figure 1A).

On September 13th, 1987, two unauthorized individuals entered the premises of the demolished institute and removed the source assembly from the radiotherapy unit in the belief that it might be of some value as scrap metal. The two individuals transported the assembly home and tried to dismantle it, unaware that inside the lead shield was a source

containing 50.9 TBq[1] of radioactive material. During their manipulations, the lead shield was ruptured, releasing the stainless steel source capsule. This contained highly radioactive $^{137}CsCl$ (cesium chloride) salt which is highly water soluble and readily dispersible. General information on ^{137}Cs and the radioactive source can be found in Table I (after IAEA, 1988).

Table I. ^{137}Cs radioactive properties and data on the source ruptured during the Goiânia radiological accident. Modified from IAEA, 1988.

^{137}Cs - general information		
Gamma emissions		0.66 MeV (84%)
Beta emissions	Maximum energies	0.51 MeV (95%)
		1.17 MeV (5%)
	Mean energy	0.187 MeV
Half-life		30 years
Specific gamma ray constant		8.9 mGy/h at 1 m per GBq
Data on the IGR's source (as of September 1987)		
Model		Cesapan F-3000®
Radioactive material		Cesium chloride
Volume		3.1×10^{-5} m^3
Mass		0.093 kg
Specific activity		0.55 TBq/g (15.1 Ci/g)
Radioactivity		50.9 TBq (1375 Ci)
Dose rate at 1 m		4.56 Gy/h

The broken pieces of the assembly were subsequently sold to a scrap metal dealer where they were left until later that week, when some of the broken pieces were sold and transferred to other junk yards. The distribution process created some of the several foci of radiation that were subsequently identified in and around metropolitan Goiânia (Figure 1H). The first night following the acquisition of the metal pieces, the dealer noticed that the stainless steel container emitted a blue glow in the dark. Curious about his new purchase he brought it inside where, using a screw driver, he proceeded to dislodge

[1] For definition of units of radiation mentioned throughout this dissertation, please refer to Appendix 2.

glowing fragments from within the container. He was astonished at his luck and thought the powder must be valuable, if not supernatural. The little gemstone-like granules and the glowing powder were distributed among his closest relatives and friends, further disseminating the radionucleide.

As a result of the dispersion of the ^{137}Cs, a number of people were exposed to ionizing radiation, and it was not long before the typical symptoms of acute radiation illness became apparent (Figure 1B through 1D). Unexpected symptoms were misinterpreted by local physicians and misdiagnosed as food poisoning, contact dermatitis, and pemphigus. To further aggravate the situation, the acute symptoms simulated those of tropical diseases. It was only when the scrap dealer's wife took the remnants of the source to a nearby office of the Sanitary Surveillance Division (SSD) that there was some suspicion concerning the possibility that the source might be responsible. Finally, a technician from the SSD and a physician from the Toxicology Information Center suggested the possibility that the material might be radioactive. On the 29th of September a nuclear physicist was called to perform preliminary measurements and immediately validated the assumption that radioactivity was involved. Immediate action was taken to isolate the area and both military and fire personnel were called upon to prevent anyone from entering any premises where radioactivity was above background.

Following the discovery of the nature of the accident, national and international emergency teams were dispatched to Goiânia to determine the extent and the level of contamination. The human consequences of this accident are summarized in Table II (after Oliveira *et al.*, 1989). In addition to obvious medical effects, severe socio-economical and psychological consequences afflicted the population. Those individuals contaminated by

radiation, and even their neighbours, were ostracized from the community and to this day still live under a stigma. Subsequent to the discovery of the accident, neighboring states refused to buy grain, milk, vegetables, and meat from the State of Goiás. This lead to major economic losses for the region.

Table II. The medical consequences of the ^{137}Cs radiological accident of September 1987 in Goiânia (Brazil).

Type of Detriment	Total
Fatalities	4
Acute Radiation Syndrome	8
Bone marrow failure	14
Local radiation injuries	28
Hospitalization	20
Contaminated individuals	
External contamination[1]	120
Internal and external contamination	129
Number of individuals monitored	112,800

[1] Clothing and shoes only.

An area of 2,000 m^2 was severely contaminated. Emergency actions were taken to clean up and control further contamination (Figure 1E and 1F). Several procedures were quickly used to bring all potential source of contamination under control. Secondly, a remediation phase was started in order to restore normal living conditions. During the clean up process, any item which could not be decontaminated was dismantled and placed into concrete-lined drums for disposal as nuclear waste. In total, 3500 m^3 of waste was generated. After extensive socio-political considerations, a temporary storage site was defined. A sparsely populated area about 23 km from Goiânia was finally chosen as the most convenient location (Figure 1G). Six open concrete platforms were built, meeting the local conditions, constraints on the construction time, and political demands. An environmental monitoring station was placed in the repository site. To date, no leakage of radioactive materials has been detected. However, due to weather conditions and high

humidity and high temperatures, the predicted corrosion of containers has been observed.

For almost a decade, Brazilian authorities have been engaged in a comprehensive decision-make process regards the final resolution as to the location and construction of the permanent repository for the radioactive wasted originated during the 1987 Goiânia radiological accident. Ongoing discussions are taking place both at the national and international levels. The discussions include the technical aspects of a permanent repository, as well as ethical and socio-political considerations. A global deliberation over all aspects involved in the decision is crucial for solving the problem on a permanent basis, as the final repository will have to last for nearly four centuries (Table III).

Table III. Radioactive ^{137}Cs contained in the waste originated during the Goiânia radiological accident.

Waste group	Volume (m^3)	Percentage of total volume	Average concentration (KBq/kg)	Decay time (years)
5	51	1.5	3.21×10^5	356
4	429	12.8	1.43×10^4	221
3	578	17.2	1.44×10^3	122
2	769	22.9	3.2×10^2	57
1	1534	45.6	26.9	0

Taking into account the packaging procedures, the volume, and average concentrations of the radionucleide, Brazilian authorities divided the radioactive waste into five groups and calculated the time it would take for each group to decay to a residual concentration level of ≤ 87 Bq/g, which is the exemption level established in Brazil (Table

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Figure 1 (next page). The Goiânia accident is the most serious radiological accident yet to occur in the Western hemisphere. (A) partially demolished premises of the IGR from where the carelessly abandoned ^{137}Cs radiotherapy unit was removed; (B,C,D) deterministic effects of radiation exposure, including radio-lesions, and skin burns induced by severe exposure to γ-rays due to contact with $^{137}CsCl$; (E) specialized emergency team undertaking clean up procedures to decontaminate severely radioactive areas; (F) the ^{137}Cs source finally shielded in a concrete container; (G) temporary storage site in Abadia de Goiás of the 3500 m^3 of radioactive waste contained in concrete-lined drums, placed on open concrete platforms; and (H) several radioactive foci in metropolitan Goiânia, which originated during the radiological accident, covering an area of 2,000 m^2. All photographs by courtesy of the Fundação Leide das Neves Ferreira, Goiânia (Brazil).


A
B
H

III, after Paschoa *et al.*, 1993). The volume of waste which will need to be confined for the longest time comprises 1.5% of the total waste volume. The largest group of radioactive waste, accounting for almost half of the total volume, already has a concentration lower than the expected exemption level and technically could be considered exempt from regulatory control.

In summary, a chain of errors lead to the 1987 radiological accident in Goiânia. In all, 249 people were exposed to ionizing radiation of cesium-137 in the worst radiation accident in the Western Hemisphere, worldwide second only in severity to the explosion of the Chernobyl reactor. The Goiânia accident has had major medical, social-economical, and physiological impacts in the community, leaving the government to grapple with the political fallout - a struggle that is not yet over. As reported by Roberts (1987), the accident, by its nature and characteristics, well illustrates "an eerie image of how wrong things can go when vigilance over radioactive material lapses."

1.2. Dosimetry

The Goiânia radiological accident had serious medical consequences, including four casualties. Notably, two weeks elapsed from the time the assembly was first dismantled to the discovery of the accident. During this time, several people were critically exposed to doses as high as 7 Gy of ^{137}Cs ionizing radiation derived from both high levels of external irradiation and severe internal contamination. The most heavily exposed individuals suffered from acute radiation sickness and radiation lesions (Figure 1B through 1D). Both dermal lesions due to beta irradiation and internal lesions caused by more deeply penetrating gamma radiation were incurred.

Dose estimation was one of the most important parameters for prognosis and treatment. However, due to the nature of the accident, precise estimates represented a major challenge. Individual exposure was very heterogeneous, and in some cases, of a fractionated character. A majority of the patients received significant whole-body and localized irradiation. Some patients had internal and external contamination from the radionucleide. All these peculiarities in extent of radiation further complicated the dose estimation for the exposed population. Nevertheless, several dosimetry techniques were used to assess the level of exposure and to provide initial information on potentially exposed individuals. The main approaches used to estimate the dose for the exposed population in Goiânia were:

1.2.1. Internal Dosimetry: Inhalation, ingestion, and absorption through wounds were the three potential pathways for internal contamination. The primary action was concentrated on determining the individual intake of cesium-137 chloride through the analysis of biological excreta. In addition to the bioassay estimates, internally deposited cesium-137 was quantitatively evaluated by using a field whole-body counter with a detection level of 9.1 KBq (247 nCi) for a counting time of 2 minutes with 95% confidence. The 70-year dose commitments for the 129 people who exhibited internal and external contamination can be seen in Table IV.

1.2.2. Biological Dosimetry: As reported by IAEA (1988), blood samples were collected from 110 individuals previously identified as having putative exposure greater than 0.1 Gy. Individual absorbed doses were estimated by means of biological dosimetry using chromosome aberration analysis. Chromosomal type aberrations, namely dicentric and

centric ring chromosomes, and acentric fragments, were scored. Dose assessments were then obtained using a calibration curve for cobalt-60 (γ-rays at a dose rate of 0.12 Gy/min) as no similar curve for cesium-137 was available at that time. The unstable chromosome aberration analysis showed that dose estimates exceeded 1.0 Gy for 29 people. However, no estimated dose exceeded 7.0 Gy (Table IV).

The incidence of chromosome aberrations followed a Poisson distribution whenever individuals had a uniform whole body exposure. Six cases, with doses ranging from 0.5-4.5 Gy, exhibited over dispersion and may indicate a partial body exposure. Nevertheless, the Poisson analysis lacked sufficient resolution to accurately discern these over dispersed findings (IAEA, 1988, Ramalho *et al.*, 1988).

Table IV. Dose commitment for 129 people exposed in the radiological accident in Goiânia (Brazil), after IAEA (1988).

Number of people	Committed dose (70 years) [Sv]
45	<0.005
42	0.005 - 0.05
33	0.05 - 1.0
4	1.0 - 2.0
2	2.0 - 3.0
1	3.0 - 4.0
1	5.0 - 6.0
1	7.0

1.2.3. External Dosimetry: Using this approach, dose estimates can be determined based on known radioactive properties of the radionucleide, dose rates and sequential reconstruction of the events that lead to exposure. External dosimetry for the exposed population in Goiânia was complicated by the complex mix of contamination, external irradiation, and accurate time factors. Moreover, the lack of precise information on individual exposure histories further complicated the dose estimations. Despite these

uncertainties, some gross assessments were made for screening purposes.

For additional details on the 1987 radiological accident with cesium-137 in Goiânia (Brazil) refer to Roberts, 1987; IAEA, 1988; Candotti *et al.*, 1988; and Oliveira *et al.*, 1989.

2. Human Radiation Exposure

2.1. Short History and Remarks

Over the past century, few issues have commanded as much public and scientific attention as those related to radiation. No single carcinogenic factor - possibly with the exception of smoking - has been so intensively studied as ionizing radiation (Larsson, 1988). Whether such interest reflects the potential benefits associated with the use of radiological and nuclear technologies, or the fear associated with the deployment of nuclear weapons, is not certain. However, since the discovery of X-rays (Roentgen, 1895) and radioactivity (Becquerel, 1896), in the latter part of the 19th century, intensive research has been carried out in order to understand and characterize the impact of ionizing radiation on human health. Since the beginning of the 20th century, it has been known that high doses of ionizing radiation produced clinically detectable harm to an exposed individual. The damage resulting from such exposure could range from eye irritation, to skin burn, to death. Some decades ago, it became evident that low radiation doses could also cause serious health effects, although such cases are of low incidence and only detectable through sophisticated epidemiological studies of large populations.

One of the first pieces in the radiation exposure-damage puzzle was provided by the demonstrated mutagenicity of X-rays in *Drosophila* (Muller, 1927). In the early 1950s the

double helix model for the DNA structure was proposed (Watson and Crick, 1953a,b) giving radiation geneticists a molecular model to explain their results. Moreover, the double helix model discovery placed the radiation problem into the field of nucleic acid chemistry. In the 1970s, the development of recombinant DNA techniques and DNA sequencing methodologies (Maxam and Gilbert, 1977; Sanger *et al.*, 1977) permitted the accurate investigation of the radiation effects on genes at a molecular level.

By the end of the 1980s, a vast amount of new genetic information had accumulated which prompted a new look at the standards governing protection against exposures to ionizing radiation and the safety of radiation sources. To date, there are significant gaps between scientific records and general public belief relating to the effect of radiation. Despite the body of scientific information regarding ionizing radiation, a clear message still emerges, which is that radiation's real and perceived risk are commonly misunderstood, and that a clear communication of the facts to the general public is yet to be achieved.

2.2. Natural and Artificial Sources of Radiation

Humans have always been exposed to ionizing radiation. Natural sources of radiation include cosmic radiation and external and internal irradiation from radioactive material in the surroundings and within the body. The absorbed dose, due to cosmic radiation, is strongly dependent on altitude. The world's average annual dose equivalent is 0.3 mSv for populations at large (UNSCEAR, 1982).

Long-lived radionucleides, such as ^{40}K, ^{87}Rb, ^{238}U, and ^{232}Th, comprise the major terrestrial sources of natural radiation. The presence of primordial radionucleides and their decay products results in external irradiation to the population. The absorbed dose is dependent

on specific environmental activity. Long-lived radionucleides in the biosphere may enter the human body through ingestion and inhalation. The most important contribution to the internal irradiation of humans from natural sources comes from the radioactive gas radon, from the decay series of ^{238}U and ^{232}Th (for detailed information see Gustafsson and Persson, 1988). Annual exposure estimates from natural sources can be found in Table V.

Table V. Global estimates of annual radiation exposure from natural sources.

Source of Exposure	Annual effective dose (mSv)*
Cosmic rays	0.39
Terrestrial gamma rays	0.46
Radionucleides in the body (except radon)	0.26
Radon and its daughters	1.3
Total	2.38

* One mSv is the currently recommended annual dose limit for members of the population at large for exposures from practices under regulatory control.

The development of radiological and nuclear technologies has contributed to the world's population radiation exposure of man-made sources. Stratospheric fallout due to nuclear test explosions, nuclear power production, medical irradiation, occupational exposure, and nuclear accidents, comprises the man-made contribution to the world's population dose of radiation. The UNCEAR (1993) report has confirmed that the normal operation of all peaceful nuclear installations contributes insignificantly to the global exposure to radiation. Gonzáles (1993) has compiled all the information on dose commitment, taking into consideration all peaceful nuclear activities, medical, occupational, and accidental exposures, and has determined global exposure to be equivalent to a few days of natural exposure (Table VI).

Radiological accidents, generally associated with complex environmental, physiological, and psycho-sociological components, have become an anticipated and not uncommon occurrence in the modern world. The potential chance of accidental exposures increases along with the development of radio-nuclear technologies. In addition to the

deliberate and calamitous dropping of the A-bomb over Hiroshima and Nagasaki in August of 1945, several severe radiological accidents have occurred in the 20th century. Two major accidents occurred at nuclear power plants - at Three Mile Island (USA) in 1979, and at Chernobyl (Soviet Union) in 1986. The accidents had a considerable impact on the public's perception of the potential danger from radiation exposure. Furthermore, accidents involving radiation sources used in medicine and industry have also attracted public attention, such as the accidents at Cuidad Juárez (Mexico) in 1982, Mohamadia (Morocco), Goiânia (Brazil) in 1987, San Salvador (El Salvador) in 1989, Soreq (Israel) in 1990, Zaragoza (Spain), and Kiisa (Estonia) in 1994 to name just a few.

Table VI. Exposure to man-made sources of radiation expressed as equivalent periods of exposure to natural sources of radiation (after Gonzáles, 1993).

Source	Basis	Equivalent period of exposure to natural sources
Medical exposure	One year of practice at the current rate	90 days
Nuclear weapons tests	Terminated practice	2.3 years
Nuclear power	Total practice to date	10 days
	One year of practice at the current rate	1 day
Severe accidents	Events to December 1993	20 days
Occupational exposures	One year of practice at the current rate	8 hours

2.3. Radiation Safety and Protection

New international standards on radiation safety and protection were discussed in a report by Gonzáles (1994a). Gonzáles highlighted the result of a joint effort towards international harmonization of radiation safety. Several organizations and committees supported the development of current safety standards which were developed following information on extensive research by scientific and engineering organizations at both the national and international levels. The newly elaborated guidelines are called BSS (International Basic Safety Standards for Protection Against Ionizing Radiation Sources). The BSS provides

guidelines for intervention in situations where individual doses approach the values in Table VII.

Table VII. Individual dose level at which intervention must be expected under any circumstances (after Gonzáles, 1994a).

a. Acute exposures

Organ or Tissue	Projected absorbed dose to the organ or tissue in less than 2 days (Gy)
Whole body	1
Lung	6
Skin	3
Thyroid	5
Lens of the eye	2
Gonads	3

b. Chronic exposures

Organ or Tissue	Annual equivalent dose rate (Sv/year)
Gonads	0.2
Lens of the eye	0.1
Bone marrow	0.4

The dose limits established by the BBS are intended to ensure that no individual is placed at unacceptable risk due to radiation exposure. Dose limits for occupational exposure, and for members of the public, can be found in Tables VIIIa and b, respectively.

Table VIII. Individual dose limits.

a. Occupational exposure

- Effective dose of 20 mSv/year averaged over 5 consecutive years;
- Effective dose of 50 mSv in any single year;
- Equivalent dose of 150 mSv for the lens of the eyes in a year; and
- Equivalent dose of 500 mSv for the extremities (hands and feet) and skin.

b. Members of the public

- Effective dose of 1 mSv in a year;
- Effective dose up to 5 mSv in a year provided that the average dose over 5 consecutive years does not exceed 1 mSv/year;
- Equivalent dose of 15 mSv in a year for the lens of the eye; and
- Equivalent dose of 50 mSv in a year for the skin

2.4. Nature and Properties of Ionizing Radiation

The term **radiation** denotes energy propagating in the form of electromagnetic waves,

photons, or in the form of subatomic particles. Ionizing radiation is a particular kind of radiation with sufficient energy to excite - in the medium through which it passes - an atom or molecule into an ion. The average ionization energy is approximately 33.7 eV (5.3×10^{-18} J) in air and, in water is about 20 eV (3.2×10^{-18} J). Only ionizing radiation with energy over 124 eV (2×10^{-17} J) is considered to be biologically significant (Gonzáles, 1994b) .

In nature, ionizing radiation can be either particulate or electromagnetic. Particulate ionizing radiation originates from particles produced from unstable nuclei. The most relevant for biological systems are α-particles, negatrons (β^-), and positrons (β^+). Electromagnetic ionizing radiation consists of high energy photons, such as X-rays and γ-rays. The first arise from the oscillation of orbital electrons, or is due to transitions of orbital electrons from a higher to a lower energy level. The latter are emitted during the transition of particles within the atomic nucleus or by reaction between fundamental particles.

Different types of radiation vary in their ionization density. The Linear Energy Transfer (LET) describes the relative amounts and distribution of ionizing radiation and excitation energy released along the track of a particle in a particular medium. For a given amount of energy absorbed, radiation which creates the denser distribution of ionization will cause the greater damage (Saddi, 1994).

The biological effect of ionizing radiation is dependent on both the absorbed dose delivered to the tissue, the dose rate, and the spatial distribution of the energy transfer, but the effect does not depend on the radiation origin (Svensson, 1988). Absorbed dose is the mean amount of energy imported from ionizing radiation by a unit mass of irradiated material at a point of interest. The current unit for the absorbed dose is expressed in Gray (Gy) which is equivalent to a 1 J/Kg. For radiation protection purposes, the absorbed dose is weighted to

take account of the effectiveness of the different radiation types, and the radiosensitivity of various organs and tissues. The resulting quantity is termed effective dose, and its unit is expressed in Sievert (Sv). For photons in the intermediate energy range, 1 Sv is approximately equal to 1 Gy (Gonzáles, 1994b).

2.5. Interaction of Ionizing Radiation with Living Matter

To consider the biological effects of radiation exposure, one needs to take into consideration that cellular material is the medium through which ionizing radiation passes. Cellular material is remarkably rich in water molecules and these molecules are immediately ionized by radiation exposure creating an abundance of OH-radicals, hydrogen atoms, and hydrated electrons. These active chemical radicals are extremely reactive and are, therefore, able to promote chemical changes in the cells. The newly formed radicals attack DNA by abstracting covalently bound hydrogen atoms or by the addition of double bonds between the DNA bases. Abstraction of hydrogen from the deoxyribose moiety results in a free radical that may undergo intra-molecular rearrangements or react with solute molecules, while reaction with the oxygen leads to an irreversible destruction of the deoxyribose, which generally leads to a chain break (Ahnström, 1988; Ward, 1988).

Direct interaction with the DNA molecule, causing the excitement of its atoms due to direct deposition of energy, is also ascribed to radiation exposure. There is a multiplicity of potential reaction mechanisms in the cellular environment which creates the potential for a large spectrum of ionizing radiation products in DNA. However, the predominance of water in biological systems suggests that species formed by the radiolysis of water are the major source of damage (Ward, 1988; Friedberg *et al.*, 1995).

Ionizing radiation induces mainly four different classes of DNA lesions, namely, single strand breaks, double strand breaks, various types of base damage, and DNA-protein cross links (Natarajan et al, 1988; Ward, 1988; Friedberg *et al.*, 1995). It has been estimated that for every 1 Gy of absorbed dose, approximately 1000 single-strand breaks, 40 double strand-breaks, and other assorted lesions are produced (Ward, 1986).

2.6. The Current Understanding of Biological Effects of Ionizing Radiation

Since the early experiments by Roentgen, it has been known that exposure to ionizing radiation produce clinically detectable harm in an exposed individual. According to our current knowledge, two main types of health effects induced by radiation exposure can be identified. The first type are called the **deterministic effects** which develop above a threshold and the severity of the effects increase with dose. In general, deterministic effects are associated with cell death (e.g., erythema, skin burn, and radio-lesions, etc.). The second type are called **stochastic effects** and are generally associated with cell modification (e.g., cancers and genetic disorders). The probability of stochastic effects increases with dose, in a manner similar to other genotoxic agents, including tobacco and some chemicals. Consequently the relative contribution of these agents can only be established through epidemiological studies. The effects of low doses of radiation can thus only be estimated in large populations. The most extensive epidemiological study in context of human radiation exposure has been the follow-up studies done over 40 year on the A-bomb survivors of Hiroshima and Nagasaki (Flakus, 1995).

The biological effects of ionizing radiation arise from damage caused to the chemical structure of the cell, particularly to DNA, where the altered structure can lead to mutation. If mutation occurs in a germ cell, it can be transferred to the progeny. If damage occurs in a

somatic cell, and the cell survives, fixed mutations could initiate malignant transformation (Gonzáles, 1994b). While radiation may induce mutation and hence contribute to both evolution and genetic variation (Pochin, 1980a,b; Denniston, 1982), the vast majority of biological effects of radiation are viewed as harmful.

Carcinogenesis is generally viewed as the most important stochastic effect of ionizing radiation. At present, carcinogenesis is assumed to be a multistage process, usually divided into three steps, namely cancer initiation, tumour promotion, and malignant progression. Ionizing radiation is assumed to be a potent initiator, rather than an effective promoter or progressor. Although, to date, it is not possible to clinically determine whether a specific malignancy has been caused by radiation exposure, radiation-induced tumours and leukemia have been detected and statistically quantified by epidemiological studies of populations exposed to ionizing radiation. With regard to germ cell events, epidemiological studies have not detected statistically significant hereditary effects of radiation exposure in humans (Larsson, 1988; Gonzáles, 1994b).

The carcinogenic effects and genetic risks of radiation have been extensively detailed in the literature and are, in a general sense, reasonably well understood. The dose-response relationship, especially at low doses, however, remains unclear. The questions of whether there is a linear or exponential dose response and whether there is a discrete threshold below which biological consequences are uncommon, remain unanswered. Similarly, we are uncertain of the extent to which such factors as LET, and dose rate, affect the outcome of exposure. Essentially, the extent of human illness arising from an increase in mutation rate due to exposure to ionizing radiation remains a critical issue (ICPEMC, 1983; UNSCEAR, 1988; Sankaranarayanan, 1993; de Serres, 1994).

3. Hypoxanthine-guanine Phosphoribosyltransferase: The Protein (HPRT), the Gene (*hprt*), and the Assay

3.1. The HPRT Protein

HPRT enzyme catalyses the formation of purine nucleotides, GMP and IMP, from PRPP and the respective purine bases. There are two major pathways for purine biosynthesis in higher eukaryotic cells, namely, the salvage reaction and the *de novo* pathway. The first consists of a much simpler and less costly reaction to the cells, in that purine nucleotides are synthesized from free purine bases formed by hydrolytic degradation of nucleic acids and nucleotides. In the second, the salvage pathway, the enzyme HPRT catalyses the conversion of guanine and hypoxanthine to guanylic and inosinic acids in the presence of PRPP (Stryer, 1988). The simplified salvage pathway can be seen in Figure 2.

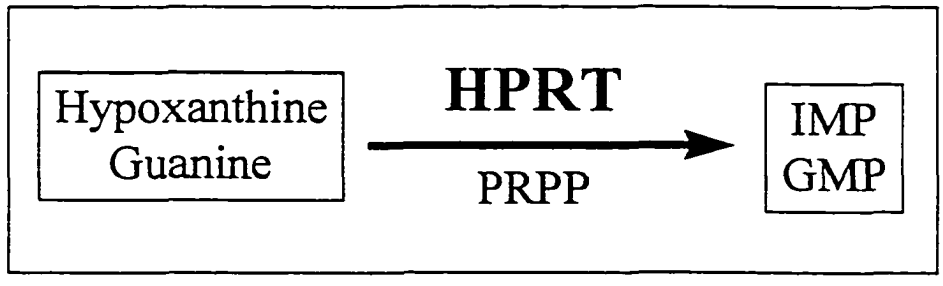

Figure 2. The HPRT enzyme is in the salvage pathway of purine nucleotides. It is responsible for recycling about 90% of all free purine bases. This enzyme catalyzes the conversion of hypoxanthine and guanine to IMP and GMP in the presence of PRPP (Stryer, 1988).

Since early description of the HPRT in the late 1960s (Seegmiller *et al.*, 1967), the enzyme and its encoding gene have received a great deal of attention in the fields of biochemistry, genetics, and medicine. This interest has resulted in the extensive investigation of both the enzyme and its respective gene, including structure, function, and

expression. HPRT deficiency is also of interest because it is associated with two clinical entities in humans. The virtual absence of HPRT enzyme activity is responsible for the Lesch-Nyhan syndrome which is characterized by the overproduction of uric acid, leading to a central nervous system disorder. The psychological profile of an individual with this syndrome includes mental retardation, spasticity, choreoathetosis, and a compulsive form of self-mutilation (Lesch and Nyhan, 1964). A partial deficiency of HPRT activity is characterized by overproduction of uric acid which may result in hyperuricemia and in an early onset of gout (Kelley *et al.*, 1967).

HPRT is a soluble cytoplasmic enzyme which does not seem to be glycosylated (Kruh *et al.*, 1981). The native molecule is a tetramer of identical subunits (Holden and Kelley, 1978). Each enzyme subunit is 217 amino acid residues long, including an acetylated alanine in the NH_2-terminal, with a molecular weight equal to 24,470 (Wilson *et al.*, 1982a,b). Studies of tissue distribution of the enzyme in humans have indicated that HPRT is constitutively present in most tissues, accounting for no more than 0.005-0.04% of total cellular protein (Melton *et al.*1981). However, cells of the basal ganglia and testes have the highest HPRT activity, while cardiac and skeletal muscle have the lowest activity. HPRT in the brain can account for as much as 0.05% of the total soluble protein. It can thus be concluded that *HPRT* concentration is 7-fold higher in the brain than in any other tissue. The mechanism and the rationale behind such a difference remains unknown (Kelley *et al.*, 1969; Wilson *et al.*, 1983; Chinault and Caskey, 1984).

Studies in human erythrocytes have shown that the HPRT protein exhibits considerable electrophoretic heterogeneity. Some posttranslational modifications may, in part, account for such heterogeneity. These posttranslational modifications include: 1)

removal of the NH_2 terminal methionine followed by acetylation of the newly formed amino-terminus; and 2) Partial deamidation of asparagine at position 106 (Johnson *et al.*, 1982; Wilson *et al.*, 1982b). Johnson *et al.* (1982) showed that modification of erythroid cells is time-dependent, starting in the reticulocyte and continuing through the erythrocyte life-time. Because of such particularities, HPRT posttranslational modifications could serve as molecular markers of enzyme aging (Wilson *et al.*, 1983).

3.2. The *hprt* Gene

Hprt is a constitutively expressed gene which encodes the enzyme hypoxanthine-guanine phosphoribosyltransferase. Pioneering studies of pedigree analysis of HPRT-deficient families suggested that the *hprt* locus was X-linked (Nyhan *et al.*, 1967). Advanced studies on segregation of HPRT-activity further confirmed the *hprt* gene to be X-linked and localized to the long arm of this chromosome at a position distal to the PRPP synthetase and proximal to the glucose-6-phosphate dehydrogenase (Becker *et al.*, 1979; McKusick, 1982). Finally, somatic cell hybridization studies localized the human *hprt* locus to Xq26-27 (Pai *et al.*, 1980) as seen in Figure 3.

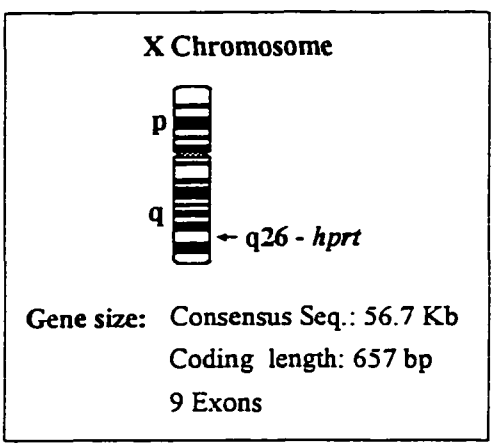

Figure 3. Ideogram of X chromosome showing the location of the *hprt* gene.

In the early 1980s the complementary DNA sequence to the HPRT messenger RNA was isolated, cloned, and ultimately sequenced. The *hprt* is a constitutive housekeeping gene, hence it is scarcely expressed with less than 10 copies of its mRNA per cell. The mature mRNA is about 1.6 kb in length with an open reading frame spanning just over 654 base pairs flanked by non-coding sequence (Jolly *et al.*, 1982 and 1983). The functional HPRT mRNA accounts for less than 3% of the genomic DNA that codes for this enzyme. The complete genomic sequence consensus of the human HPRT stretches across 56,736 bases (Edwards *et al.*, 1990). Like most eukaryotic genes, the *hprt* gene is a mosaic of exons separated by long intervening introns. The interrupted coding sequence splits the gene into nine protein-coding exons dispersed over a very large region of DNA. Extensive posttranscriptional events remove the eight introns during the processing of the primary transcript to yield a much smaller mature mRNA. Three probable pseudogene sequences have been found in humans, and mapped to chromosomes 3, 5, and 11. Aside from being intronless, these pseudogenes are relatively smaller than the functional gene (Chinault and Caskey, 1984; Patel *et al.*, 1984).

During RNA splicing, introns are removed from the primary transcript by a system that recognizes short consensus sequences at the exon-intron boundaries and within the intron (Lewin, 1994). In the *hprt* gene the splice junctions are highly conserved and they all comply with the GT-AG rule. All donor sites start with the dinucleotide GT and the acceptor sites ends with the dinucleotide AG.

The promoter region of the *hprt* gene is located in its 5'-end and it has a TATA-less promoter that lacks the typical 5' transcriptional regulatory sequence element know as the TATA or CAAT boxes. Instead, the *hprt* core promoter has extremely GC-rich

sequences, including five GC hexanucleotide motifs (5'-GGCGGG-3') upstream from the transcription start point. These motifs are at -214, -202, -183, -178, and -167 and are known to bind the cellular transcription factor Sp1 (Kim *et al.*, 1986; Rincón-Limas *et al.*, 1991). Additionally, there is a motif between -204 and -195 of sequence 5'-GGGGCGGGGC-3' that is similar to the consensus sequence for the high-affinity binding site of Sp1 (Kadonaga *et al.*, 1986). Several other regulatory elements, similar in homology to the enhancer core sequences of the promoter of Simian Virus 40 (SV40), are displayed in the 5' region of the *hprt* gene (Kim *et al.*, 1986; Rincón-Limas *et al.*, 1991).

In addition, there are multiple potential transcription start sites in the region immediately downstream from the last GC box. The major functional transcription initiation sites are found at approximately position -93 and -88, upstream from the ATG translational start codon (Kim *et al.*, 1986). A study of the functional characterization of the *hprt* gene promoter demonstrated that consensus sequences from positions -219 to -122 direct a relatively high level of expression and also exhibit bi-directional activity. Moreover, an upstream regulatory element, spanning positions -570 to -388, negatively modulates this gene expression in position-dependent and orientation-independent fashions (Rincón-Limas *et al.*, 1991). Based on the *hprt* promoter sequence, all the basal transcriptional factors are needed for transcription, including the TFIID, the initiator (Inr), and possibly an additional factor to bind to the Inr and anchor the complex of RNA polymerase II general factors. The role of TFIID is not yet clear, but presumably TFIID interacts with the Sp1 in order to recognize the promoter. In the *hprt* gene, the putative Inr element is located between -2 and +2, with a 5'-TTAT-3' consensus sequence, which provides the positioning element for the transcriptional complex (Lewin, 1994).

The *hprt* mRNA is 1.6 kb long and has a methylated cap at the 5'-end. The methylated cap is the first structure to be recognized by the ribosomes and cap-binding proteins during the process of translation initiation. The binding of the ribosome and associated factors to the methylated cap unwind the mRNA leader region enabling the ribosome to migrate to its binding site. With respect to translation efficiency of the *hprt* mRNA, the two guanines, at positions -8 and -3 respectively, are the two most important because they are the binding sites for the 40S ribosomal subunit (Lewis, 1994).

At the 3'-end of the noncoding region of the *hprt* gene, the putative sequence element, believed to be the signal associated with post-transcriptional cleavage and polyadenylation of eukaryotic mRNA, can be found. The putative element is postulated to be a stretch of adenine and uracil (AAUAAA). For the *hprt* gene, the putative element is located 13 bases upstream from the site of polyadenylation (Wilson *et al.*, 1983) and its exact location is at base pair 42075, following the numbering system of Edwards *et al.* (1990).

3.3. Overview of the 6-Thioguanine Selection Assays

Since the early 1970's, cultured human cells have been known to have some spontaneous resistance to purine analogues. The spontaneous resistance was postulated to be induced by known mutagens such as X-rays and N-methyl-N'-nitro-N-nitrosoguanidinine. Furthermore, the possible enumeration of variant cells resistant to purine analogues *in vitro* was addressed as a potential method of detecting mutagenesis and as a useful method to detect induced phenotypic changes that could be associated with cancer (DeMars, 1974).

Earlier chemical information on the enzyme HPRTG (hypoxanthine-guanine phosphoribosyltransferase) confirmed that the conversion of purine analogues to nucleotides inhibited the growth of normal fibroblasts. However, those cells lacking a functional enzyme could proliferate in the presence of the analogues by re-synthesizing nucleotides through the *de novo* pathway, using previously synthesized nucleotides (DeMars, 1974). The enzyme deficiency was putatively associated with spontaneous somatic cell mutation of the *hprt* gene arising *in vivo*. Consequently, the *hprt* locus has been extensively used for studies of mutations in cultured somatic cells that were able to grow and form colonies in the presence of a purine analogue as a selective agent.

3.3.1. The autoradiographic assay. The autoradiographic method was developed to quantify the variant cells using T-lymphocytes in the presence of 6-thioguanine and it was proposed for use as a direct mutagenicity test for humans. In the autoradiographic assay, cells lacking a functional HPRT were detected by their ability to incorporate [^{3}H]thymidine in the presence of 6TG and to therefore be detected by autoradiography (Strauss and Albertini, 1979).

Initially it was assumed that only variant cells lacking HPRT activity could undergo division in the presence of selective agents. The lack of direct proof that the 6-thioguanine-resistant (TG^R) cells were mutants, and the relatively high mutant frequency of variant cells, made it difficult for the autoradiography assay to find general acceptance. The high estimates of mutant frequencies reported in early studies (Strauss and Albertini, 1979) were later attributed to lymphocytes undergoing division while in the peripheral blood. These presumed TG^R cells, no longer in G_0 phase, are able to incorporate the radioactive label in the presence of 6TG prior to their death. Further studies reported that

most of the thioguanine-resistant lymphocytes detected by autoradiography were indeed mutant cells. In addition, the studies reported an excessive variability observed with different methods, suggesting that the use of the autoradiography assay should be limited to studying large populations of cells (Dempsey and Morley, 1983). The basic procedure of the autoradiographic assay has been extensively modified (Morley *et al.*, 1983; Vijayalaxmi *et al.*, 1984; Everson *et al.*, 1985). Some of the modifications include cytometric cell sorting (Amneus and Erickson, 1986), and the freezing and thawing of the cells prior to stimulation with PHA (Albertini, 1982). A different version of the assay, using 5-bromodeoxyuridine incorporation and Hoechst 33258 dye to identify the 6TG-resistant cells with fluorescence microscopy, was also developed (Ostrosky-Wegman *et al.*, 1987).

The limitations of the autoradiographic assay became the rationale for improving clonal expansion of putative mutant cells allowing for further molecular analysis. Firstly, towards this end the capacity to directly clone and propagate T-lymphocytes *in vivo* (Paul *et al.*, 1981) was developed. Secondly, mass T-cell culturing techniques were developed by using basic growth media enriched with T-cell growth factors and feeder cells. T-cells were cloned in a 96-well plate in the presence and absence of 6TG as the selective purine analogue. Each well was monitored microscopically for cell growth. Cell densities were maintained under optimal conditions by splitting the cell colonies as necessary. The availability of cultured T-lymphocytes, introduced by the modifications to the former assay, facilitated the investigations of the genetic mechanisms underlying 6TG resistance and variability of mutations among the T-cell populations (Albertini *et al.*, 1982).

3.3.2. The clonal assay. This *hprt* assay involves the cloning of the T-cell fraction of the mononuclear cells from peripheral blood. Cells are cultured in 96-well microtitre plates in the presence of phytohaemagglutinin (PHA). PHA is a mitogenic plant lectin which causes an *in vivo* stimulation of T-lymphocytes by cross-linking the CD3 complex on the T-cell receptors. This non-specific stimulation, associated with the cytokine inter-leukin-2 (IL-2) and accessory ("feeder") cells, induces a T-cell proliferative response comparable with the antigen driven systems. Furthermore, the T-cell response is controlled by a down-regulation mechanism, where, in the absence of further stimulation, IL-2 receptor expression on responding T-cells is short-lived. To maintain high expression of the IL-2 receptors and proliferative activity of the T-cell clones, further PHA stimulation is required. PHA alone initiates cell division by passing G_0 T-lymphocytes into G_1. However, IL-2 and "feeder" cells are essential to make the stimulated T-cells go into S-phase and commit to cell division (Fleischer, 1983; Hadden, 1988; Beare *et al.*, 1993). Under these conditions, T-lymphocytes undergo clonal expansion and clones can be scored, following approximately a 14-day incubation. The addition of 6-thioguanine to the growth medium selectively kills the normal cells and the *hprt* mutant cells form colonies. Both cloning efficiencies per clonable cells, that is, in the presence and absence of 6TG, can be calculated from the negative component of the Poison distribution.

The T-cell clonal assay is indeed more demanding of resources, time, and money than the autoradiography assay. Nevertheless, it is superior in that the use of T-lymphocytes and their reasonably simple expansion enable full characterization of each individual clone. With the aid of both molecular and biochemical techniques, the mutant phenotype can be determined and the mutational spectrum investigated (Rossi *et al.*, 1992;

Beare *et al.*, 1993). Moreover, molecular analysis of T-cell receptor rearrangements was developed which provided the means to investigate the clonal origin of mutant T-lymphocytes (Nicklas *et al.*, 1986). The rationale is that *in vivo* clonal expansion of mutant cells could lead to spuriously high estimates of true mutant frequency of independent mutations. The comparison of TCR patterns identifies sibling mutants, and thus the observed mutant frequency can be corrected accordingly. The clonal approach, in conjunction with the TCR molecular analysis, is particularly useful in cases where an exceptionally high mutant frequency has been observed (Nicklas *et al.*, 1988).

The clonal *hprt* assay using T-lymphocytes from the mononuclear cell fraction from the peripheral blood has been extensively used to monitor the human population for somatic mutations. Culture conditions vary between individual laboratories. Nevertheless, the observed mutant frequencies are quite similar. A considerable body of data from healthy as well as individuals exposed to several mutagens, ranging from cigarette smoke to ionizing radiation, has been accumulated (for detailed information see Curry, 1993 and references therein). Moreover, a large database including information on the *hprt* sequence and characterized mutations has been collected. As a result, an electronic database is now available, including software to facilitate the analysis and comparisons of laboratories' results (Cariello *et al.*, 1992, Cariello, 1994).

4. T-lymphocytes

The term **lymphocyte** includes an array of distinct cells which have a lymphoid origin and which are specifically associated with the recognition and response of foreign antigens. In mammals, the specificity of the immune response is mediated by two broad

classes of lymphocytes, namely, the T- and B-lymphocytes. These two cell lineages derived their common name from the tissue they were first shown to mature. T-lymphocytes maturing in the thymus and B-lymphocytes maturing in the bursa of Fabricius in birds (there is no equivalent of such an organ in mammals where early stages of B-lymphocyte maturation occurs in the bone marrow). Lymphocyte subsets are quite different in their function, although they all appear similar in morphology (Table IX). B-lymphocytes can undergo clonal expansion and are responsible for humoral immunity for they are the only cells capable of producing antibodies. These particular lymphocyte subsets are not further discussed in the present dissertation.

The second major lymphocyte subset is the T-cells (T-lymphocytes) which are the main subject of this chapter. T-cells, like B-lymphocytes, are also antigen responsive through cell-mediated (cellular) immunity but are not directly related to antibody production. Basically, T-cells recognize and respond to cell surface-associated antigen by secreting cytokines. Cytokines are hormones that promote the proliferation and differentiation of the T- and B-lymphocytes, macrophages, and granulocytes.

There are at least three lymphocyte classes, including three T-lymphocyte subsets. Each group is defined by the cell surface markers and the immediate function. The fundamental knowledge of the lymphocyte classes is summarized in Table IX (after Abbas *et al.*, 1994).

Table IX. Summary of lymphocyte classes found in peripheral blood.

Class	Functions	Receptor	Common Phenotypic Markers	Percentage of Total Lymphocytes		
				Blood	Lymph node	Spleen
B-lymphocytes	Antibody production	Immunoglobulin	Fc receptors, class II MHC	10-15	20-25	40-45
T-lymphocytes Helper	- Stimuli to B cell growth and differentiation - Macrophage activation	αβ heterodimers	$CD3^+$, $CD4^+$, $CD8^-$	50-60	50-60	50-60
Cytolytic	- Lysis of virus-infected cells, tumor cells, and allografts -Macrophage activation	αβ heterodimers	$CD3^+$, $CD4^-$, $CD8^+$	20-25	15-20	10-15
γδ T-cells	- Unknown	γδ heterodimers	$CD3^+$, $CD4^-$, $CD8^-$	1-10	ND**	ND
Supressor Cells*	- down-regulate immune response	Unclear	Unclear ($CD8^+$)	ND	ND	ND
Natural Killer Cells	- Lysis of virus-infected cells and tumor cell - Antibody dependent cellular cytotoxicity	Unknown	Fc receptor for IgG (CD16)	~10	Rare	~10

* It is yet unclear if these so-called "supressor cells" are a distinct subpopulation of T-lymphocytes.
** Not determined.

4.1. Phases of Immune Response Related to T-Lymphocytes

The word **immunity** is derived from the Latin word *immunitas* which referred to the exemption from various civic duties and legal prosecution offered to Roman senators during their tenures in office. Biologically, immunity means protection from diseases, more specifically, infectious diseases.

The modern definition of immunity is a reaction to foreign substances, mainly proteins. A collective T-lymphocyte response to the introduction of foreign substances

comprises the cell-mediated immunity. Cell-mediated immune response involves the production of specialized cells that react with foreign antigens on the surface of host cells or antigen presenting cells (APCs). Essentially, the cell-mediated immune response may be putatively divided into three phases, as follows:

4.1.1. The cognitive phase which consists of the binding of the antigens to specific receptors on the surface of T-cells. T-lymphocytes express receptors which recognize only short peptide sequences that are previously bound to another cell, through the MHC complex.

4.1.2. The activation phase which is the sequence of events induced in T-cells as a consequence of antigen recognition. Lymphocytes undergo two major processes after antigen recognition. Firstly, T-cells proliferate, leading to clonal expansion of antigen-specific cells that amplify the immune response. Secondly, T-lymphocytes differentiate from cells whose primary function is cognitive to cells that eliminate foreign antigens.

4.1.3. The effector phase is the stage in which antigen-specific activated T-cells eliminate the antigen. Several effector functions require the participation of other, non-lymphoid cells, and defense mechanisms.

4.2. T-Lymphocyte Development

All T-lymphocytes are derived from a common lymphoid precursor. The common progenitor originated from pluripotent haematopoietic stem cells that also give rise to a common myeloid precursor associated with the production of all the remaining blood cells, including the mononuclear lineages and the erythrocytes. The human immune system is composed of 2×10^{12} lymphocytes and approximately 70-80% are T-cells which are found

in large numbers in specialized lymphoid organs such as the thymus, lymph nodes, the spleen, gut-associated lymphoid tissues, and the appendix.

T-cell development and differentiation occur in the thymus. However, the thymic stem cells are formed in the haematopoietic tissues of bone marrow and fetal liver. The stem cells migrate from the tissue of origin to the fetal thymus via the peripheral blood. Immature T-cells arrive at the fetal thymus in waves and are committed to the T-lymphocyte lineage. As yet immature cells do not express TCR or any accessory molecules and, therefore, are unable to recognize antigens and perform any effector function. Once in the thymus cortex, T-cell precursors (thymocytes) reach a very high rate of mitosis, giving rise to multiple progeny. While undergoing division, thymocytes come in contact with a diversity of non-lymphoid cells, including thymic epithelial cells, macrophages, dendritic cells, and nurse cells. The interaction of the young T-cells with the above mentioned cells is a primordial step in T-lymphocyte maturation.

The non-lymphoid cells in the thymus cortex express a unique class of surface molecules called the MHC (Major Histocompatibility Complex). There are two classes of such molecules, namely class I MHC and class II MHC. The interaction of maturing thymocytes with MHC molecules provides the means for the selection of the mature T-cell repertoire. The selection processes begin after developing T-cells first express randomly generated TCR molecules. The diversity of the T-cell repertoire arises from a variety of combinations of the V, J, and D (β chain only) gene elements. Further variability is achieved by addition or deletion of nucleotides around the junction regions. Increased diversity of rearrangement and recombination of the TCR gene creates an array of distinct TCR molecules which facilitate the recognition of an infinite number of antigens.

Two major events shape the T-cell repertoire of antigen-binding receptor. The events are positive and negative selection. Positive selection is responsible for eliminating all cells which cannot bind to class I or class II MHC within the thymus. Positive selection ensures that all mature T-cells will be able to interact with self MHC. However, positive selection leaves behind harmful self antigen-specific T-lymphocytes. In order to eliminate the possibility of autoreactive receptor combinations, negative selection, possibly secondary to the positive selection, eliminates all T-cell clones that bind with high affinity to self-antigens associated with self MHC molecules. There are two putative components associated with the negative selection. One component is the clonal deletion where self reacting T-lymphocytes are prevented from reaching the periphery, with pathogenic consequences. A second component is the clonal anergy, where the self reacting T-lymphocytes are inactivated. The outcome of the overall selective process is a population of mature, self MHC-restricted, and self-tolerant T-cells. In the process, over 95% of the cortical thymocytes die before reaching the medulla.

4.3. T-Cell Cycle

While a T-lymphocyte completes its trajectory from the thymus cortex to the medulla it reaches its maturity and then leaves the thymus to enter the blood stream. At this stage of development, T-cells are known as naïve or virgin cells for they have never encountered their specific antigen. The naive T-cells have a high expression of a surface molecule, known as L-selectin. This ligand binds to specialized endothelium in lymph nodes (or in other lymphoid organs), mediating these organs as a homing signal for naïve T-cells.

Once a naïve T-lymphocyte encounters its specific antigen in a lymphoid organ, it becomes activated and enters its cell cycle. Immediately after recognition of the antigen, the activated T-cell responds by increasing the expression of several of its surface proteins. The T-cell then remains a resident of the lymphoid organ. Nevertheless, the T-cell's progeny may differentiate to become either effector or memory cells and return to the peripheral blood. Memory cells have a long life span, perhaps as long as 20 years in humans, and they promote an effective secondary immune response to be mounted rapidly if the same antigen is encountered again.

T-cells are continuously recirculating between blood and lymph. This dispersion ensures that an antigen is exposed to its specific T-lymphocytes. After antigen presentation by an antigen presenting cell (APC), T-cell clonal expansion begins and an immune response develops. The effector phase of a specific immune response requires the participation of various defense mechanisms, including the complement system, phagocytes, inflammatory cells, and cytokines. In order to be recognized by a T-lymphocyte, the antigen must be in a peptide form for T-lymphocytes only recognize linear determinants of a primary amino acid sequence. Therefore, APCs have to process the antigen prior to its presentation to the T-cells. This processing includes the partial hydrolysis of the foreign protein and binding of the antigen pieces to the MHC molecules at the cell surface.

4.4. The T-Cell Receptors

The T-cell receptor (TCR) is a complex of several integral plasma membrane proteins expressed on the surface of a mature T-lymphocyte. The TCR is responsible for

specific antigen recognition, as well as recognition of self MHC (a phenomenon known as MHC-restricted recognition). The T-cell receptors are dimers consisting of two disulfide-linked chains, noncovalently associated with the CD3 protein to form a functional TCR-CD3 complex at the cell surface.

The molecular nature of the TCR has been elucidated. The conventional TCR expressed in most T-lymphocytes is called the αβ receptor which consists of two polypeptide chains, namely α and β. The primary function of the αβ receptor is to bind processed peptide antigen complexed to MHC molecules and polymorphic determinants of self MHC molecules on the surface of APCs. Another less common subset of T-lymphocytes bears polypeptide chains called γ and δ chains, making the γδ receptor. The functions and attributes of this receptor are unknown. Both αβ and γδ TCR are produced from TCR genes, uniquely expressed in T-lymphocytes. The functional TCR genes undergo intense somatic rearrangement throughout T-cell development. The rearrangements give rise to an incredible diversity of TCRs, making possible the limitless antigen specificity in T-cells. The highly diverse nature of the rearranged TCR sequences allows for the use of these genes as markers for identifying clonally related T-lymphocytes.

For a detailed review on T-lymphocyte development, cell cycle, and T-cell receptors, the reader is referred to references in Abbas *et al.* (1994), Kendrew (1994), Curry (1993), Roitt *et al.* (1985).

CHAPTER II . Human Micronucleus Counts Are Correlated with Age, Smoking, and Cesium-137 Dose in the Goiânia (Brazil) Radiological Accident

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Abstract

A random sample of 276 people representing control, direct exposure, and probable indirect exposure in the Goiânia, Brazil radiological accident was examined using micronuclei as indicators of cytogenetic damage. The Goiânia subjects were analyzed for interactions of age, lifestyle, and ionizing radiation dose. Increases in micronucleus frequencies were most strongly correlated with the dose of ionizing radiation, but age, alcohol consumption, and smoking habits also effected micronucleus frequencies. Despite these additional influences, micronucleus frequencies can be useful as biological dosimeters.

1. Introduction

On September 13, 1987, a radiological accident involving ^{137}Cs occurred in Goiânia, Brazil. An abandoned radiotherapy source containing 50.9 TBq (1375 Ci) of cesium-137 was removed from its protective housing by two men who sold the metal pieces to a scrap metal dealer. The source casing and cesium-137 itself was distributed to several residences and public places. At least 100 people received external or internal exposure with dose rate 4.6 Gy/h at 1 m (IAEA 1988; Ramalho *et al.* 1988; Natarajan *et al.* 1991a,b). Most of the affected subjects were exposed over a protracted period at varying dose rates. Exposures ranged from acute to chronic. Total dose was predominantly due to external contamination, but this was frequently accompanied by ingestion of the nuclide (Ramalho *et al.* 1988). Doses ranging up to 7 Gy (700 rad) produced multiple cases of radiation sickness and four fatalities.

Cytogenetic changes, such as chromosome aberrations, sister chromatid exchanges, and micronuclei have frequently been utilized as indicators of biological damage. Ionizing radiation is a well established inducer of chromosome aberrations and micronuclei which can be used as a biological radiation dosimeters (Stenstrand, 1985). The documented dose-response relationships for ionizing radiation suggest that the micronucleus assay may be one of the easiest and most reliable tests for the determination of radiation sensitivity and exposure (Prosser *et al.* 1988; Ban *et al.* 1991). Micronuclei are formed during cell division from acentric fragments or lagging chromosomes and are readily detected in interphase cells as free intracytoplasmatic bodies (Hogstedt, 1984; Stenstrand, 1985; Savage, 1989; Littlefield *et al.*, 1989; Baunchinger and Braselmann, 1990). However, it remains unclear how factors such as age and lifestyle interact with ionizing radiation in the production of cytogenetic changes. We have examined this question in people exposed during the Goiânia accident.

2. Material and Methods

2.1. Sampling and Determining Micronucleus Frequencies

Peripheral blood was obtained 1 year after the Goiânia radiological accident from 276 random donors from Goiânia, Brazil classified into five exposure groups (Table X) . Age, sex, smoking, and alcohol consumption were recorded for each subject. Two groups (the foci in Table X) received unknown exposure to ionizing radiation, and it was our secondary goal to estimate the dosage they received using micronucleus frequencies.

Table X. Statistical data for the five sampled exposure groups.

Variables	Direct Exposure	Staff	Focus 2	Focus 9	Control
Sample size	75	18	66	50	65
Mean dose (rad)	77.6	4.4	unknown	unknown	unexposed
Mean micronucleus frequency	1.5×10^{-2}	1.8×10^{-2}	1.1×10^{-2}	8.7×10^{-3}	9.9×10^{-3}
Micronucleus frequency SD	1.0×10^{-2}	3.4×10^{-3}	3.6×10^{-3}	4.8×10^{-3}	6.6×10^{-3}
Mean age (years)	24.1	38.6	37.7	38.3	32.7
Female	50.7%	61.1%	63.6%	64.4%	69.7%
Smokers	36.0%	61.1%	33.3%	40.0%	37.9%
Drinkers	26.7%	55.6%	30.3%	42.0%	45.5%

Dose was estimated from urine and faeces bioassay, whole-body counting, and frequency of chromosome aberrations at the time of the accident (IAEA, 1988; Ramalho *et al.*, 1990). Foci refer to the two main foci of contamination. Members of these foci may have received indirect exposure to ionizing radiation. Control individuals were selected from parts of the city away from the accident and dispersed cesium-137. Staff refers to health, environmental, and safety workers involved in the treatment of exposed individuals and the clean-up of radiation sources.

Blood samples were drawn into vacutubes containing sodium heparin. A 1.5 ml volume of peripheral blood from each donor was cultured using the microculture method of Fenech and Morley (1986), with modifications. Cells were cultured in a 37°C, 10% carbon dioxide humidified atmosphere in Ham F-10 modified medium (Cultilab) containing 20% foetal calf serum, 2mM L-Glutamine (Gibco), and 5 µg/ml phytohaemagglutinin (Welcome). After 44 hours, cytochalasin-B (Gibco) was added to a final concentration of 3 µg/ml in order to block cytokinesis. This compound is an inhibitor of microfilament assembly that prevents cytoplasmic division after nuclear division has occurred (Fenech and Morley, 1986). After 72 hours, the cells were harvested by centrifugation and treated with hypotonic solution (0.075 M KCl), fixed in 3:1 methanol:acetic acid, and then distributed to 10 microscope slides. Cells were stained with 4% Giemsa solution for 15-20 minutes to make nuclei visible.

Scoring of micronucleus frequencies was performed for binucleate cells only. A minimum of 1000 cells were scored for each blood sample. The four criteria of

Countryman and Heddle (1976) were used for micronucleus scoring: 1) diameter less than one third of the nucleus; 2) non-refractility; 3) staining not darker than the nucleus; 4) location within three or four nuclear diameters of the nucleus, never touching the nucleus. Questionable micronuclei were not counted if unique to a non-control group of individuals (after Countryman and Heddle, 1976).

2.2. Data Analysis

The first step was to transform the micronucleus frequencies by the natural logarithm to reduce non-linear relationships among the frequencies and to reduce non-normality in the data, both assumptions of the analyses used. Two individuals were removed from the analysis because they were extreme outliers and thus had a disproportionate influence on the results: individual 7 was the only individual with a cell with five micronuclei and individual 213 was much older than the other representatives of her exposure group.

The second step was to use a multivariate analysis of variance (MANOVA, Dillon and Goldstein, 1984) to examine if the exposure groups differed in the frequency of cells with a single, two, three, and four micronuclei. Differences were illustrated using canonical discriminant analysis (CDA; Dillon and Goldstein, 1984).

Differences discovered could have been due to radiation exposure, smoking, age, alcohol consumption, sex differences, or the number of binucleated cells counted. The third step was to reduce differences contributed by non-radiation sources. Correlations between non-radiation sources and the micronucleus frequencies (represented by the canonical variates of the CDA) were measured to identify confounding sources of

micronucleus frequency differences. Once identified, their effect was reduced by using the residuals of a linear regressions between the canonical variates and the confounding sources of micronucleus frequency differences in the remaining analyses. Value for sex, smoking, and alcohol use, as used in the analyses, are explained in the Appendix 1.

The fourth step was to examine if the residual differences were significant among groups by examining the 95% confidence ellipses of the residual canonical variates. Since the effects of non-radiation sources were reduced, differences from the control group could only have been due to radiation exposure or unmeasured factors.

The final step was to measure the correlation of dose of radiation with the residual canonical variates (representative of the micronucleus frequencies) for those groups with known radiation doses. These correlations were then used to predict the dose received by the two foci of unknown exposure.

3. Results

The five exposure groups differed in micronucleus frequencies (MANOVA, $p<0.01$). The main difference (first canonical variate or CV 1) was in the frequency of cells with a single micronucleus and the minor differences (second canonical variate or CV 2) was in the frequency of cells with two or three micronuclei (Table XI). These two differences were correlated with age, smoking, alcohol use, and the number of cells counted and thus were not readily interpretable in terms of dose alone (Table XII). Age (Eq. 1), number of cells sampled (Eq. 2), smoking (Eq. 3 and 4), and alcohol consumption (Eq. 4) all had a positive additive effect upon micronucleus frequencies. Smoking was the strongest confounding factor. Differences in micronucleus frequencies contributed by

these sources were reduced by using the residuals of equations 1-4 in the remaining analyses.

$$CV\ 1 = -0.841 + 0.250 \times \log_e(age + 1) \quad (1)$$

$$CV\ 2 = -7.26 + 1.04 \log_e(cells + 1) \quad (2)$$

$$CV\ 1 = -0.114 + 0.300 \times Smoking \quad (3)$$

$$CV\ 2 = -0.177 + 0.141 \times Smoking + 0.172 \times Alcohol \quad (4)$$

When confounding variation was reduced, the exposed staff and direct exposure individuals had higher micronucleus frequencies than the non-exposed control individuals (Figure 4). Focus 2 individuals were not significantly different from control individuals in micronucleus frequencies. Focus 9 individuals were not significantly different from control individuals on the main axis (CV 1) but differed on the minor axis (CV 2) in that they had fewer cells with two or three micronuclei than the control individuals.

Table XI. Canonical discriminant analysis of micronucleus frequencies among the exposure groups.

Discriminant	CV 1	CV 2
Eigenvalue	70.52%	24.33%
Eigenvectors		
$\log_e$(freq. 1 micronucleus)	1.062	-0.329
$\log_e$(freq. 2 micronuclei)	0.099	0.833
$\log_e$(freq. 3 micronuclei)	-0.114	0.487
$\log_e$(freq. 4 micronuclei)	-0.171	0.337

Eigenvalues represent the proportion of total variation explained by each axis. Eigenvectors represent the relative importance of the different frequencies to each axis.

Table XII. Pearson's rank correlations between raw CV scores and the various predictors.

Discriminant	CV 1	CV 2
Dose	0.356	0.203
Age	0.147	0.063
Sex	-0.015	0.026
Smoking	0.191	0.092
Alcohol	0.057	0.108
Number of cells sampled	0.029	0.148

Variation among individuals within the exposure groups was considerable (Table X). As a result, attempts to elucidate a robust, quantitative model to predict individual dosage from canonical variate score were a failure. However, increased dose of ionizing radiation was positively correlated with increased micronucleus frequency (Table XIII). While focus 2 individuals were not significantly different from control individuals, the micronucleus frequencies of focus 9 individuals were correlated with a radiation dose lower than control individuals.

Table XIII. Pearson's rank correlations between residual CV scores and the various predictors.

Discriminant	CV 1	CV 2
Dose	0.343	0.205
Age	-0.030	0.045
Sex	0.003	0.055
Smoking	0.000	0.000
Alcohol	-0.033	0.000
Number of cells sampled	0.050	0.005

Exposure to ionizing radiation also resulted in a change in the covariance of the two canonical variates (Figure 4). For non-exposed, increase in the frequency of cells with a single micronucleus covaried with decrease in the frequency of cells with two or three micronuclei. For exposed, there was a positive covariance, i.e., increase in the frequency of any number of micronuclei covaried with increases in the frequencies of the other numbers of micronuclei.

4. Discussion

There are four concerns for the use of the micronucleus assay as a biological dosimeter: (1) the sensitivity of the assay which depends upon the amount of ionizing radiation needed to produce detectable levels of micronuclei; (2) the temporal stability of

radiation induced micronuclei; (3) the time period before micronuclei induced from non-radiation sources (noise) confound the radiation induced micronucleus frequencies.

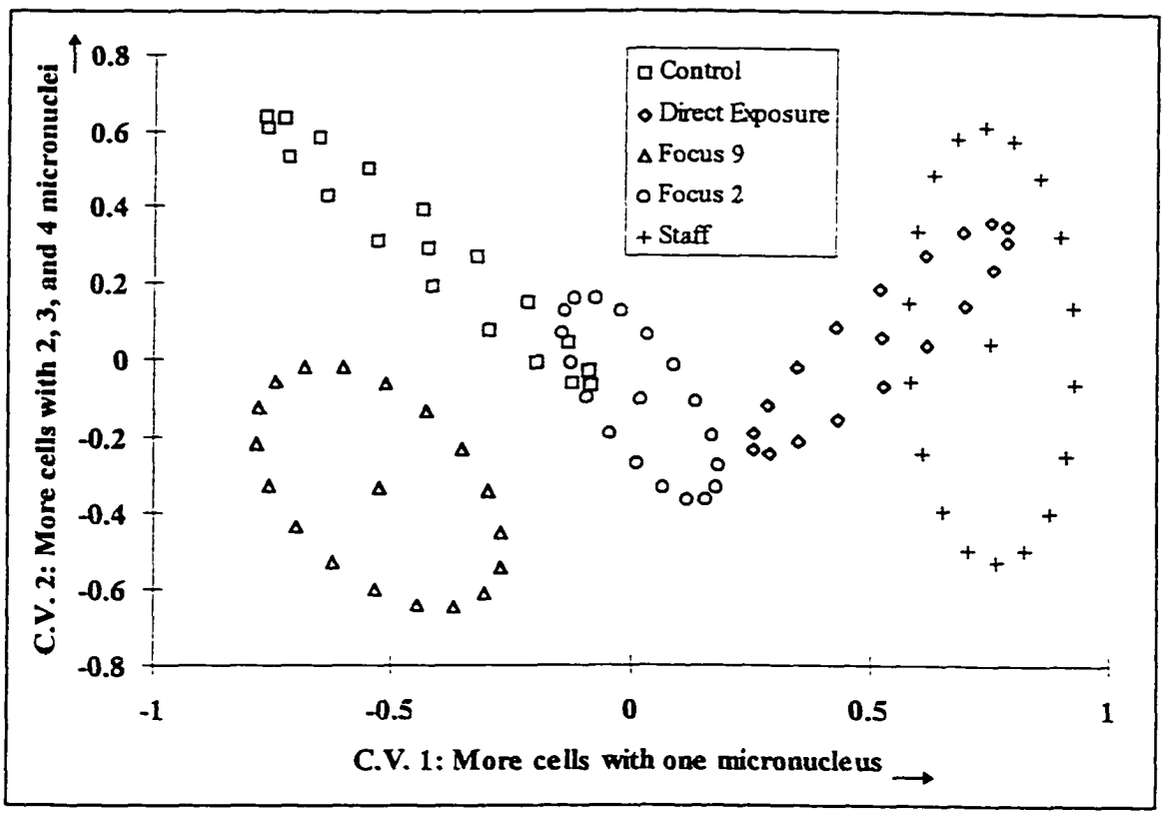

Figure 4. Exposure groups differed in micronucleus frequencies after the effects of smoking, age, number of cells sampled, and alcohol consumption were factored out. Ovals represent 95% confidence. Exposed groups (staff and direct exposure) had higher frequencies of cells with single micronucleus.

In a re-analysis of published studies, Bauchinger and Braselmann (1990) concluded that the micronucleus assay was reliable for screening radiation exposure exceeding 0.2 Gy of a low linear energy transfer radionucleide. Individuals from the two foci may thus have been exposed to low levels (< 20 rad) of ionizing radiation, a possibility that the micronucleus assay cannot assess.

Blood samples taken 1 year after the Goiânia accident revealed dose related increases in micronucleus frequencies indicating that micronucleus frequencies can be used to estimate the dose received by individuals from the two foci. Even after 1 year, control

and exposed individuals still differed in micronucleus frequencies. However, smoking and other factors confound the relationship between dose and micronucleus frequencies and their influence must therefore be taken into account. The results for Focus 9, which had micronucleus frequencies lower than control group, suggests that additional, unknown factors can influence micronucleus frequencies such that the accumulation of micronuclei from non-radiation related causes may, with time, obscure the radiation induced frequencies. As micronuclei result from chromosome breakage or aneuploidy (Erexson *et al.*, 1991; Stenstrand, 1985), induced micronuclei tend to decrease in frequency with time as chromosomal damage leads to cell death or micronuclei are lost during cell division. It seems likely that cells with more chromosomal damage, and hence more micronuclei, would be lost at a higher frequency than those with less damage. We suggest therefore that the relative prevalence of cells with a single micronucleus in differentiating the exposed from the control group is the result of the preferential loss of cells with more than a single micronucleus that occurred during the year between exposure and blood sampling. The examination of micronuclei at a later period, e.g. 5 years, is needed to determine whether this trend will continue until the control and exposed can no longer be distinguished.

While the micronucleus assay served to differentiate between groups of people exposed to different amounts of ionizing radiation, we found it impossible to robustly predict individuals dosage from the micronucleus frequencies even after confounding sources of variation were reduced. All the predictive models examined were weakened by the large degree of unexplained variation in micronucleus frequencies among individuals.

We note that there is a positive correlation for exposed individuals having cells with one micronucleus also having cells with two or more micronuclei (Figure 4). In other words, if exposed persons have cells with a single micronucleus, they are more likely to have additional cells with multiple micronuclei. This positive correlation was not observed in the control groups (Figure 4).

The micronucleus assay is inexpensive, quick, and relatively simple to perform. Even 1 year after exposure, we have shown it to have strong predictive utility for high dose exposures as long as comparison to control individuals is used. Unfortunately, the micronucleus assay can not detect low levels (<0.2 Gy) of ionizing radiation. Additionally, prediction of individual dosage (true dosimetry) is not possible. We conclude however that the micronucleus assay is a useful biological dosimeter for human populations even if blood samples are taken a year after the exposure to ionizing radiation. While the human lymphocyte micronucleus assay cannot replace detailed chromosomal analysis for precise estimations of radiation doses, it can be used as a quick predictive model of exposure for screening purposes.

CHAPTER III. Monitoring *Hprt* Mutant Frequency Over Time in T-Lymphocytes of People Accidentally Exposed to High Doses of Ionizing Radiation

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Abstract

Modern technologies have provided the opportunity to monitor mutations in people *in vivo*. The subjects of this study were accidentally exposed to cesium-137 in a radiological accident that occurred in September 1987 in Goiânia, Brazil during which more than 150 people received doses greater than 0.1 Gy and as high as 7 Gy. The objective of this study was to determine how long the *hprt* mutant T-cells in the peripheral blood contribute to mutant frequency by examining the time-course of the T-lymphocyte response to ionizing radiation. This report describes the results obtained over a period of 2.3 to 4.5 years subsequent to the accident, from 11 subjects with doses ranging from 1 to 7 Gy, and from 9 control subjects selected from the same population. The mean lnMF (±SE) of the control group was 2.5(±0.2) + $\ln 10^{-6}$. The exposed group had a significantly increased mutant frequency; the mean lnMF (±SE) were 3.3(±0.3) + $\ln 10^{-6}$, 2.8(±0.2) + $\ln 10^{-6}$, and 2.3(±0.2) + $\ln 10^{-6}$, in the years 1990-1992 respectively. Based on the decline of mutant frequency and using Buckton's models [Buckton *et al.*, (1967): Nature 214:470–473], we demonstrated that mutant T-cells have a short term memory with a half life of 2.1 years. This relatively short half-life limits the effective use of the *hprt* assay as the method of choice to monitor past exposure. The data also demonstrate a positive correlation with age, and an inverse correlation with plating efficiency.

1. Introduction

On September 13, 1987 a highly radioactive cesium-137 source (50.9 TBq or 1375 Ci) [International Atomic Energy Agency (IAEA), 1988] was removed from an abandoned radiotherapy clinic in Goiânia, Brazil. The source was subsequently dismantled and sold as scrap metal. During the ensuing weeks, numerous people were exposed, both externally and internally, to significant levels of ionizing radiation. Upon the discovery of the accident, over 120,000 people were screened. Ultimately more than 150 people were found to have received doses ranging from 0.1 to 7 Gy (Ramalho *et al.*, 1988; IAEA, 1988). Four of those exposed

died from acute radiation syndrome within four weeks of their admission to hospital.

This study describes the genetic monitoring of several of the higher dose victims of the accident over time. The purpose of this investigation was to conduct a longitudinal study of radiation-exposed individuals to understand the frequency of mutation and the fate of mutant T-lymphocytes in the peripheral blood over time. This report comprises the results obtained from 11 of those accidentally exposed individuals with doses ranging from 1 to 7 Gy. The *hprt* mutant frequency measurements were performed at yearly intervals over a period of 2.3 to 4.5 years subsequent to the exposure and were compared to a nine-individual control group selected from the same population who were currently healthy and had no history of radiation exposure.

The subjects received a combination of external beam irradiation, skin contamination and internal contamination. Several methods were used to estimate the dose received, including: 1) internal dosimetry - bioassay and whole body monitoring; 2) cytogenetic dosimetry - the estimation of doses by chromosomal aberrations analysis; 3) external dosimetry - dose estimates from reconstruction and on the basis of radiation effects. The details on how dose estimates were determined is contained in the IAEA (1988) report on the accident.

Biodosimetric results obtained one year after the accident, using the methods of somatic "null" mutation at the glycophoryn A locus and chromosome translocations detected by *in situ* hybridization (these assays were not readily available at the time of the accident), are in general agreement with the results originated immediately after the accident using dicentric chromosome aberrations (Straume *et al.*, 1991).

Ionizing radiation has potential mutagenic effects on DNA (Ahnström, 1988; Natarajan *et al.*, 1988) causing somatic mutations, and therefore may exert a carcinogenic stimulus, even

at low levels of exposure (Upton, 1986; Larsson, 1988). Such biological effects have been extensively studied in a variety of species and experimental systems (Sankaranarayanan, 1982; Fuscoe *et al.*, 1992a,b). The detection and characterization of *in vivo* somatic cell mutation occurring in humans is made possible by the use of the *hprt* T-cell clonal assay, allowing enumeration of 6-thioguanine-resistant (TG^R) T-lymphocytes from peripheral blood. The mutants are selected by their ability to form colonies in the presence of the purine analogue 6-thioguanine (6TG) [Albertini *et al.*, 1982; Morley *et al.*, 1983; Albertini, 1985; Albertini *et al.*, 1985; Cole *et al.*, 1988; Hakoda *et al.*, 1988b].

Several investigators have used the *hprt* clonal assay to assess background mutant frequencies in normal healthy individuals, as well as subjects with genetically determined DNA repair deficiencies (Cole *et al.*, 1988; McGinniss *et al.*, 1989; Cole *et al.*, 1991; McGinniss *et al.*, 1990; Tates *et al.*, 1991; Davies *et al*, 1992; Branda *et al.*, 1993; Steingrimsdottir *et al.*, 1993; Finette *et al.*, 1994; O'Neill *et al.*, 1994; Robinson *et al.*, 1994); to measure the mutant frequencies of A-bomb survivors (Hakoda *et al.*, 1988a,c; Hakoda *et al.*, 1989; Nakamura *et al.*, 1991), and of individuals exposed to radiation due to occupational (Tompa and Sapi, 1989; Seifert *et al.*, 1993), accidental (Ostrosky-Wegman *et al.*, 1990; Natarajan *et al.*, 1991a,b), therapeutic (Messing and Bradley, 1985; Nicklas *et al.*, 1990; Sala-Trepat *et al.*, 1990; Ammenheuser *et al.*, 1991; Kelsey *et al.*, 1991; Caggana *et al.*, 1991, 1992), or environmental exposure (Bridges *et al.*, 1991). The *hprt* clonal assay also has been used to determine the mutant frequency in individuals exposed to cigarette smoke (Branda *et al.*, 1992; Ammenheuser *et al.*, 1994), and as an assay for biological dosimetry of radiation-exposed subjects (Nakamura *et al.*, 1990, 1991). *In vitro* measurement of *hprt* mutant frequencies has also been reported (Sanderson and Morley, 1986; O'Neill *et al.*, 1990a,b).

We have examined the genetic effects of the accidental radiation exposures that occurred in Brazil, using the clonal *hprt* T-cell assay (Skandalis *et al.*, 1997). That study revealed radiation-dose dependent response in individuals exposed to ionizing radiation. In the present paper, we address what happens to the *in vivo hprt* mutant frequency over time.

2. Material and methods

2.1. The *hprt* Assay

We have followed the method described by Curry *et al.* (1993). Briefly, in the *hprt* clonal assay, T-lymphocytes are cloned under selective and non-selective conditions and the mutant frequency is calculated from the ratio of the two cloning efficiencies, assuming a Poisson distribution in both T-lymphocyte populations (Morley *et al.*, 1985; Albertini *et al.*, 1990a; McGinniss *et al.*, 1990). To determine cloning efficiency (CE) the negative natural log of the ratio of the number of negative wells to the total wells is divided by the number of cells per well (Albertini *et al.*, 1982). In the presence of 10^{-5} M of the selective agent (6-thioguanine) mutant cells lacking a functional *hprt* gene are able to proliferate by synthesizing guanine from preformed bases, a reaction known as the purine *de novo* pathway (Ammenheuser *et al.*, 1988). In contrast, wild type T-cells metabolize the 6TG and are killed. The *hprt* gene is X-linked (Cox and Masson, 1978), which has the advantage that both males (hemizygotic) and females (functionally hemizygotic) can be studied (Chinault and Caskey, 1984; Melton *et al.*, 1984; Stout and Caskey, 1985).

Mononuclear cells were recovered from subjects exposed to high doses of cesium-137 ionizing radiation during the radiological accident in Goiânia (Brazil). Samples were collected at yearly intervals beginning in 1990 (2.3 years post-exposure), 1991 (3.3 years post-

exposure), and 1992 (4.5 years post-exposure). Blood was also collected from a control group in 1990. Utilizing questionnaires, it was found that individuals in the control group were currently healthy, had no remarkable past medical histories and had not recently received significant diagnostic radiation exposure for either medical or dental purposes. They were not in the vicinity of the radioactive accident, had no history of previous chemical exposure, and were not currently taking any medications. Blood samples were collected from both exposed and control groups on a voluntary basis.

Samples were collected using 10 ml-Leucoprep (Becton-Dickinson) tubes and MNCs were collected following the manufacturer's directions. MNCs were counted and diluted to 10^7 cells/ml in 50% calf serum (Professional Diagnostics), 10% DMSO (Sigma), and 40% RPMI 1640 (Hyclone). Cells were placed above the liquid phase of liquid nitrogen (LN_2) such that the temperature dropped at approximately -1°C/min until they reached -80°C, after which samples were stored under LN_2 and shipped from Brazil to Canada on either dry ice (1990) or in the gas phase of LN_2 (1991, 1992). Cells were kept under LN_2 until required. Cells were then treated as described by Curry *et al.* (1993).

Taking into consideration the possible effect that adverse factors would have on mutant frequency, we have done several repeat experiments in order to more accurately determine the mutant frequency. In general there was little variability in the repeated cloning efficiencies, and the mutant frequencies.

2.2. Statistical Method

Due to the circumstances of the accident, only a limited number of subjects were exposed to high doses of ionizing radiation of ^{137}Cs ($\geq$ 1 Gy). Consequently these analyses

were restricted to only a relatively small number of individuals. As the distribution of mutant frequencies is skewed, our statistical analysis is based upon the natural logarithms of mutant frequencies corrected by plating efficiency (lnMF) [Henderson *et al.*, 1986; Tates *et al.*, 1991; Branda *et al.*, 1993]. Logarithmic transformation was applicable because this sample has a heteroscedastic variance and a constant coefficient of variation (Zar, 1984), exhibiting an increasing variability of mutant frequency.

Exposed and control results were compared using analysis of variance to assess differences in lnMF, cloning efficiency, dose and age. Regression analyses were performed to examine the relationships between lnMF, cloning efficiency, dose, and age, adjusting for age and plating efficiency. Excel 5.0 (Microsoft) was used to perform these analyses.

3. Results

This report describes the measurement of the *hprt* mutant frequencies in the blood of a total of 11 individuals accidentally exposed to high doses of cesium-137. A total of 23 determinations were performed on non-identical cohorts of 7, 10, and 6 at yearly intervals over a period of 2.3 to 4.5 years subsequent to their exposure (Tables XIV- XVI). For comparison, a control group (Table XVII) of 9 local individuals was studied in parallel with the first year samples to determine the background mutant frequency in the Goiânia population. The means of the natural logarithmic mutant frequencies (±SE) during those three years were 3.3 (±0.3) + $\ln 10^{-6}$ (1990), 2.8(±0.2) + $\ln 10^{-6}$ (1991), and 2.3(±0.2) + $\ln 10^{-6}$ (1992) and are plotted in Figure 5. For the control group the mean of the natural logarithmic mutant frequency (±SE) was 2.5(±0.2) + $\ln 10^{-6}$. We took into consideration the reports of a wide variation in cloning efficiency and, therefore, have performed repeat experiments (data not shown). We found that,

for the same individual, the variation in the cloning efficiencies was not statistically significant and that the mutant frequencies were similar. For each individual sample we referred to the mutant frequency from the experiment with the higher plating efficiency, as it represents the most confident measure.

Table XIV. Biostatistical information and analysis of 1990 sampling of ionizing radiation-exposed individuals in Goiânia (Brazil).

Code #	Sex	Age	Dose (Gy)	Pos.[a]	Tot. wells[b]	Pos.[b]	PE (%)	MF/PE ($x10^{-6}$)	Upper[c]	Lower[c]	lnMF/PE ($+\ln 10^{-6}$)
B16	M	35	1	83	1280	54	18.9	22.8	46	11	3.13
B17	F	31	1	32	760	25	6.1	55	122	25	4.01
B64	M	16	1.3	42	88	1	8.2	13.9	56	3	2.63
B1[d]	M	49	2.1	71	760	28	15.4	24.4	51	12	3.19
B18[d]	M	15	2.9	117	264	11	31.3	13.6	28	7	2.61
B9[d]	M	43	3	67	200	7	14.3	24.9	55	11	3.21
B19[d]	M	39	7	38	748	58	7.3	110	187	65	4.70
n		7									
Mean		32.6	2.6				14.6	37.8			3.35
SD_{n-1}		12.9	2.11				8.8	34.73			0.75
SE		4.91	0.8				3.33	13.13			0.28
95% C.I.		9.62	1.56				6.52	25.73			0.56

[a]Non-selective plates.
[b]Selective plates.
[c]95% confidence interval ($x10^{-6}$).
[d]After Skandalis *et al.*, 1997.

Table XV. Biostatistical information and analysis of 1991 sampling of ionizing radiation-exposed individuals in Goiânia (Brazil).

Code #	Sex	Age	Dose (Gy)	Pos.[a]	Tot. wells[b]	Pos.[b]	PE (%)	MF/PE ($x10^{-6}$)	Upper[c]	Lower[c]	lnMF/PE ($+\ln 10^{-6}$)
B126	M	36	1	108	568	32	27.6	21.05	39	11	3.05
B105	F	32	1	65	592	30	13.8	37.73	72	20	3.63
B81	M	17	1.3	104	240	6	26	9.73	24	4	2.27
B101	M	16	1.6	102	704	15	25.3	8.5	23	3	2.14
B135	M	50	2.1	141	560	21	44.2	8.64	18	4	2.16
B86	M	24	2.9	107	456	18	27.2	14.76	30	7	2.69
B103	M	16	2.9	101	1352	39	25	11.77	27	5	2.47
B83	M	44	3	100	576	21	24.5	15.13	32	7	2.72
B162	M	46	4.4	60	376	13	12.5	28.18	62	13	3.34
B82	M	40	7	77	472	27	17.1	34.46	63	19	3.54
n		10									
Mean		32.1	2.72				24.3	19			2.8
SD_{n-1}		13.1	1.85				8.95	10.89			0.56
SE		4.14	0.59				2.83	3.44			0.18
95% C.I.		8.12	1.15				5.55	6.75			0.35

[a]Non-selective plates.
[b]Selective plates.
[c]95% confidence interval ($x10^{-6}$).

Table XVI. Biostatistical information and analysis of 1992 sampling of ionizing radiation-exposed individuals in Goiânia (Brazil).

Code #	Sex	Age	Dose (Gy)	Pos.[a]	Tot. wells[b]	Pos.[b]	PE (%)	MF/PE ($x10^{-6}$)	Upper[c]	Lower[c]	lnMF/PE ($+\ln 10^{-6}$)
B189	M	37	1	74	152	3	16.2	12.28	35		
B201	F	33	1	132	400	26	38.8	17.33	24		
B194	F	32	1.1	34	112	1	6.5	13.8	126		
B192	M	17	1.6	134	144	3	39.9	5.28	14		
B193	M	25	2.9	76	368	3	16.8	4.88	24		
B190	M	41	7	50	384	5	10	13.04	46		
n		6									
Mean		30.8	2.4				21.4	11.1			
SD_{n-1}		8.63	2.35				14.5	4.98			
SE		3.5	0.96				5.9	2.03			
95% C.I.		6.91	1.88				11.6	3.98			

[a]Non-selective plates.
[b]Selective plates.
[c]95% confidence interval ($x10^{-6}$).

Table XVII. Biostatistical information and analysis of non-exposed individuals in Goiânia (Brazil).

Code #	Sex	Age	Dose (Gy)	Pos.[a]	Tot. wells[b]	Pos.[b]	PE (%)	MF/PE ($x10^{-6}$)	Upper[c]	Lower[c]	lnMF/PE ($+\ln 10^{-6}$)
BC10[d]	M	20	0	59	1088	14	12.2	10.6	37	3	2.36
BC11	M	25	0	125	248	8	35.1	9.34	21	4	2.23
BC2[d]	F	21	0	79	488	9	17.7	10.5	21	5	2.35
BC33[d]	F	4	0	112	640	8	29.2	4.31	15	1	1.46
BC4[d]	F	42	0	116	312	19	30.9	20.3	37	11	3.01
BC5[d]	M	50	0	105	920	33	26.4	13.8	29	7	2.62
BC66[d]	F	41	0	73	528	27	15.9	32.9	62	17	3.49
BC67[d]	F	35	0	89	648	20	20.8	15.1	34	7	2.7
BC8[d]	F	35	0	97	464	16	23.5	15	32	7	2.71
n		9									
Mean		30.3					23.5	14.65			2.55
SD_{n-1}		14.1					7.55	8.17			0.56
SE		4.71					2.52	2.72			0.19
95% C.I.		9.23					4.93	5.34			0.37

[a]Non-selective plates.
[b]Selective plates.
[c]95% confidence interval ($x10^{-6}$).
[d]After Skandalis *et al.*, 1997.

Multi-factorial ANOVA using age, cloning efficiency, and dose (for the exposed group alone) resulted in no significant differences among the four groups. We expanded our analysis to consider potential interactions among dose and age using multiple regression procedures. Due to the limited number of subjects in our study, no interaction among these variables was

found. The multiple regression equations with these variables did not predict mutant frequency and no precise conclusions could be drawn (data not shown).

We removed the age effect on mutant frequencies by using either the formula reported by Branda *et al.*, (1993) [lnMF=1.46 + 0.018Age - R^2=0.015; p=0.001] or the formula for the Brazilian control group. With either correction, there was a significant difference in mutant frequency among the three cohorts (p=0.004 and p=0.021, respectively).

Relationships between mutant frequency, cloning efficiency, age, and dose were determined as follows:

3.1. Dose and Time

Positive correlation between mutant frequency and dose was observed in the samples collected 2.3 and 3.3 years after exposure. However, this positive correlation was lost as the mutant frequencies returned to control levels by 4.5 years post-exposure. Using regression analysis, there was not significant relationship between mutant frequency and exposure, probably due to the limited sample size of this study. A comparison between these two groups of correlation coefficients indicates that even 3.3 years after radiation exposure a discrete dose effect remains present when one uses the *hprt* system to determine mutant frequency on exposed groups (Figure 5). The relationship between lnMF and dose is described by the equations:

$$1990: \quad \ln MF = 2.769 + 0.224 \text{ dose} \quad (R^2=0.39;\ p=0.13) \qquad (1)$$

$$1991: \quad \ln MF = 2.448 + 0.130 \text{ dose} \quad (R^2=0.18;\ p=0.21) \qquad (2)$$

$$1992: \quad \ln MF = 2.307 - 0.003 \text{ dose} \quad (R^2=0;\ p=1) \qquad (3)$$

Our data demonstrate that decreased correlation between mutant frequency and dose is

accompanied by an increased influence of age. We thus conclude that, 2.3 years after exposure, the age effect has been obscured by the dose effect. However, by 4.5 years post-exposure the age effect predominates and the dose effect is no longer apparent.

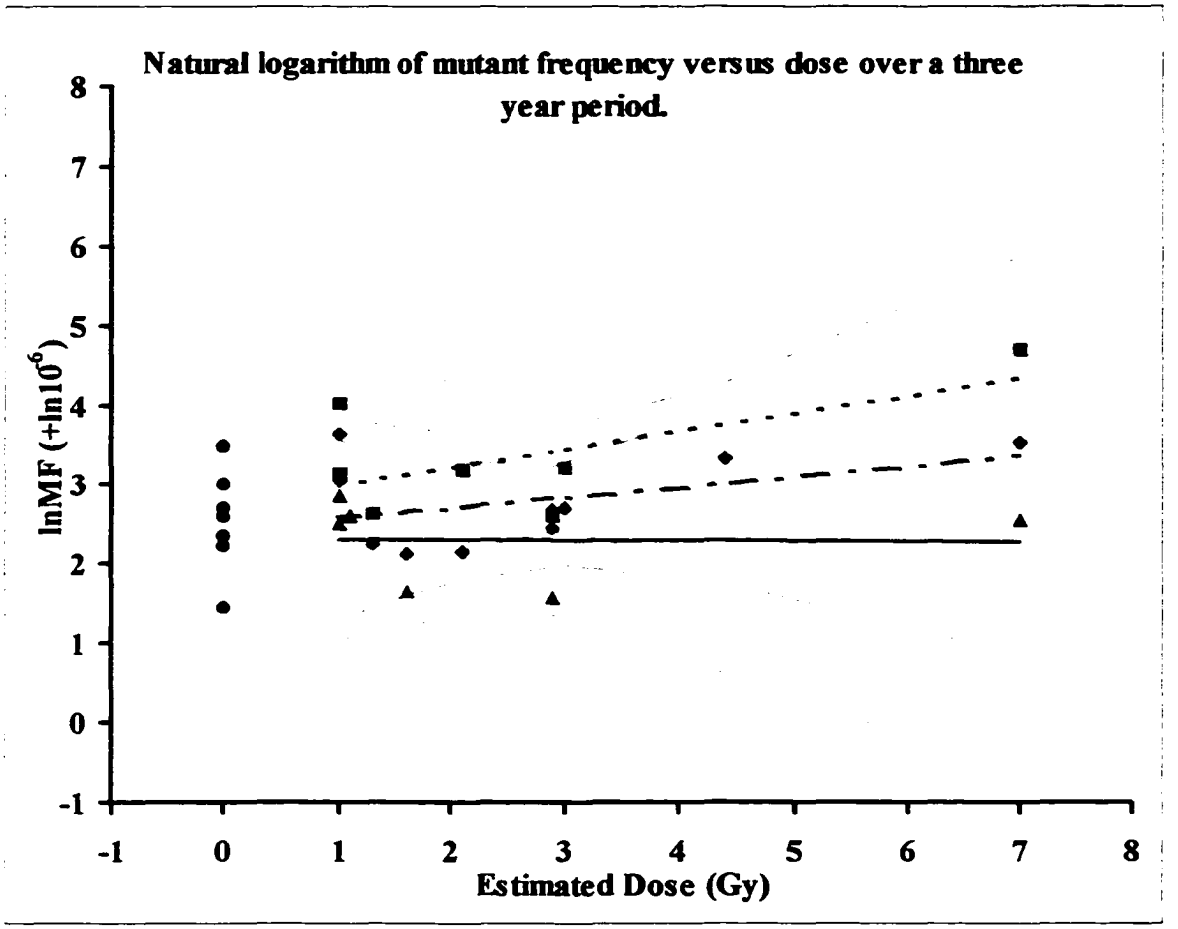

Figure 5. This figure depicts the relationship between lnMF and estimated dose over a three year period for subjects exposed to high levels of ^{137}Cs during the Goiânia radiological accident. The mean lnMF (±SE) for the control group is 2.3 (±0.2) + $\ln 10^{-6}$. The slopes for the regression lines of the exposed groups are +0.224, +0.13, and -0.003 for the three years studied, respectively. Legend key: (●) Control group; (■, – – –) 1990 regression line and confidence limits; (◆; — - — -) 1991 regression line and confidence limits; (▲; ——) 1992 regression line and confidence limits.

The modest radiation-induced elevation of *hprt* mutant frequency, seen at 2.3 years post-exposure, has been driven by two determinations, B17 and B19. If these points are removed from the data analysis the dose-response is lost and equation (1) becomes lnMF=2.941 + 0.006dose (R^2 =0; p=1). Unfortunately, the TCR assay was not available when

these experiments were carried out as the issue of clonality was only beginning to be addressed in the scientific community. Therefore, no clonal determinations were performed for these data points and they could be seen as outliers. Our concerns have somewhat alleviated since the sequencing work done on 100 mutants collected in 1991 has shown a spectrum comprised of unique mutations with less than 3% identical mutants. Nevertheless, we appreciate that this does not preclude the occurrence of clonal expansion accounting for the large mutant frequency reported.

We used Buckton's model (Buckton *et al.*, 1967) to estimate the mean half-life of T-lymphocytes bearing mutations at the *hprt* gene. The equation for this model is:

$$P_t = P_c + P_o e^{-bt} \qquad (4)$$

where P_t is the mean of lnMF for the 1990 samples; P_c is the mean of lnMF of the control group; P_o is the mean of lnMF for the 1992 samples; t is the time-course between P_t and P_o being 2.2 years. The inverse of b is the mean half-life of the T-cells bearing mutation at the *hprt* locus. With this model we calculate a mean half-life for T-lymphocytes mutated at the *hprt* gene as of 2.1 years.

3.2. Age

In this study mutant frequency shows a positive correlation with age as shown in Figure 6 and described by the equations:

1990: lnMF = 2.454 + 0.028 age (R^2=0.23; p=0.28) (5)

1991: lnMF = 2.247 + 0.017 age (R^2=0.16; p=0.25) (6)

1992: lnMF = 0.762 + 0.050 age (R^2=0.65; p=0.05) (7)

Control: lnMF = 1.551 + 0.033 age (R^2=0.69; p=0.006) (8)

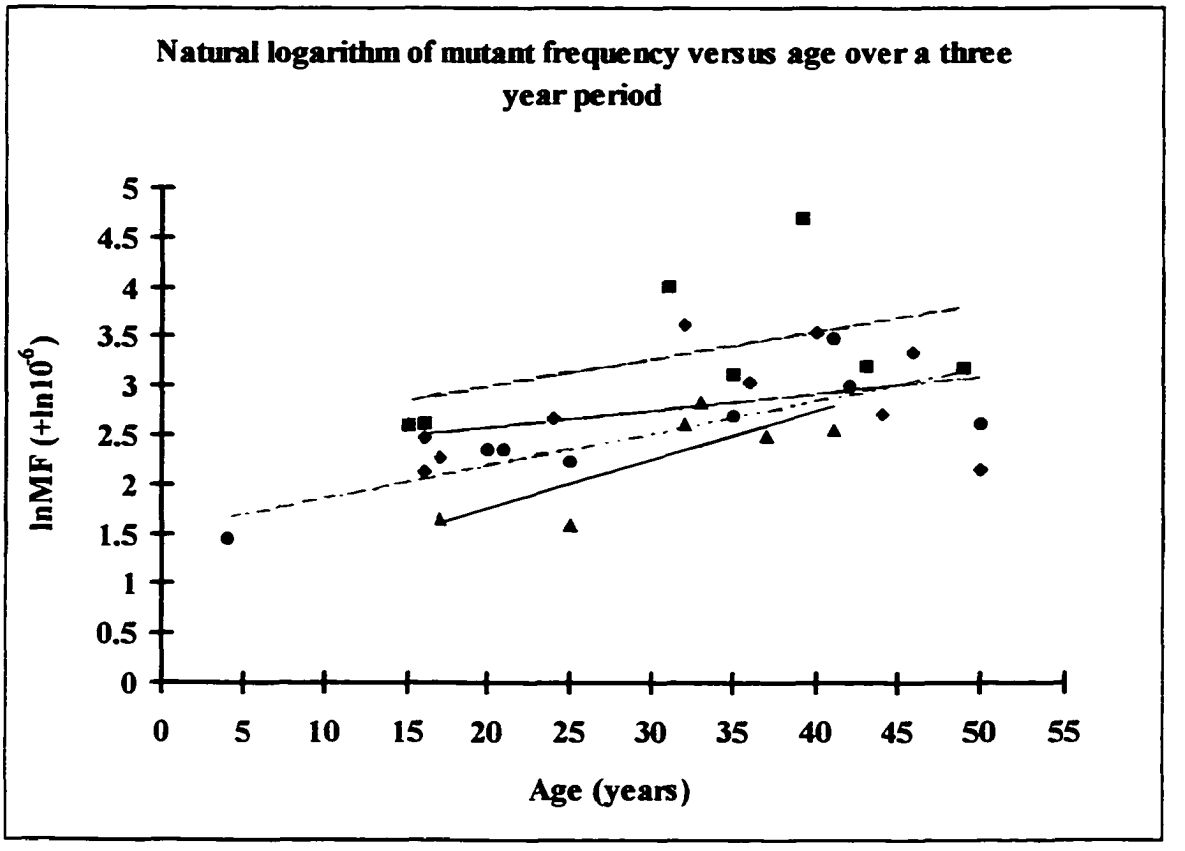

Figure 6. Regression lines indicating the effect of subject age on the lnMF at the *hprt* locus of individuals exposed to ionizing radiation over a three year period and the control group. Legend key: (●, — - - -) Control group; (■, - - -) 1990 regression line and confidence limits; (◆; — - — -) 1991 regression line and confidence limits; (▲; ——) 1992 regression line and confidence limits.

The age range in the three cohorts is 15 to 49 years, 16 to 50 years, and 17 to 41 years for 1990, 1991, and 1992 respectively. The nine healthy control individuals used in this study, with age ranging from 4 to 50 years, have an increase in mutant frequency of 3.3% per year.

3.3. Cloning Efficiency

As previously reported, cloning efficiency correlates negatively with mutant frequency (O'Neill *et al.*, 1987; Cole *et al.*, 1988; Messing *et al.*, 1989; Cole *et al.*, 1991; Tates *et al.*, 1991). We also report such a correlation for both the exposed samples and the controls as plotted in Figure 7.

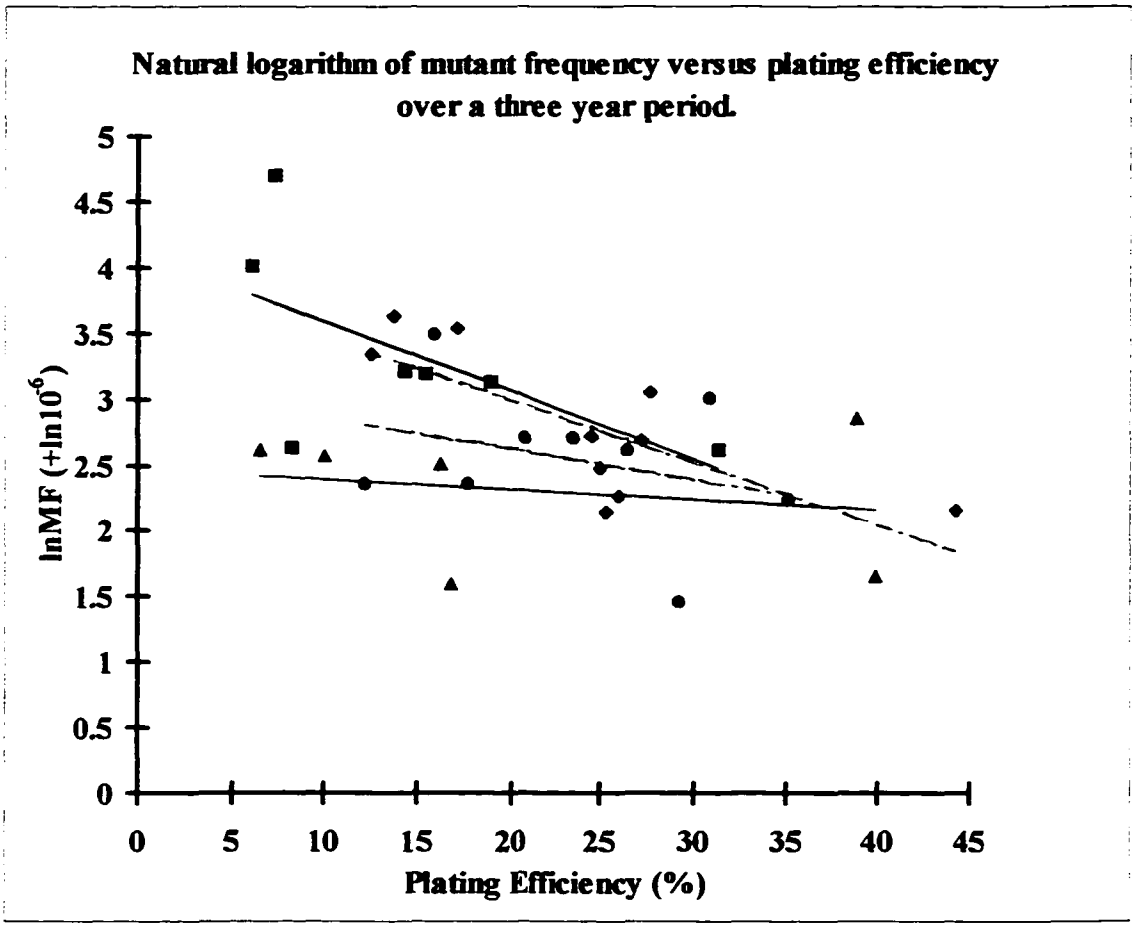

Figure 7. Regression lines showing the effect of plating efficiency on the lnMF of individuals exposed to ionizing radiation during the radiological accident in Goiânia (Brazil). Legend key: (●, — - - —) Control group; (■, – – –) 1990 regression line and confidence limits; (◆; — - — -) 1991 regression line and confidence limits; (▲; ——) 1992 regression line and confidence limits.

The negative correlation of cloning efficiency with mutant frequency is also described by the equations:

$$1990: \quad \ln MF = 4.11 - 0.05PE \ (R^2=0.37;\ p=0.15) \qquad (9)$$

$$1991: \quad \ln MF = 3.96 - 0.05PE \ (R^2=0.57;\ p=0.01) \qquad (10)$$

$$1992: \quad \ln MF = 2.46 - 0.01PE \ (R^2=0.04;\ p=0.70) \qquad (11)$$

$$\text{Control:} \quad \ln MF = 3.09 - 0.02PE \ (R^2=0.10;\ p=0.41) \qquad (12)$$

4. Discussion

As pointed out by Seifert *et al.*, (1993) when an assay is used to verify the mutagenic effect of any mutagen, one must be particularly careful about stating mutation frequencies, as mutagenic treatments can have different effects on cell viability and cloning efficiency. Several groups have addressed many of the factors that can potentially affect individual mutation frequencies (Grosovsky and Little, 1985; Cole *et al.*, 1988; O'Neill *et al.*, 1989; Tates *et al.*, 1991; Davies *et al.*, 1992; Seifert *et al.*, 1993; O'Neill *et al.*, 1994; Khaidakov and Glickman, 1996). To some extent, therefore, mutation frequency can be considered to be dependent upon experimental conditions (e.g., medium factors such as serum and growth factors), methods of cryopreservation, lifestyle factors (e.g., diet, smoking, and alcohol use), exposure to chemical and physical mutagens, genetic constitution, individual DNA repair capacity, age-related effects on cloning efficiency, and cell cycle and natural life span.

Our examination of the *hprt* mutant frequencies for those accidentally exposed to ionizing radiation reveals two major findings: 1) The *hprt* mutant frequency was higher in those exposed to high doses of ^{137}Cs ionizing radiation during the Goiânia radiological accident than in the control group obtained from the same population. This is in agreement with the results reported by Natarajan *et al.*, (1991b) on a sub-set of these high dose exposed individuals and our work (Skandalis *et al.*, 1997). 2) The *hprt* mutant frequency of the exposed subjects decreases gradually over time, showing the *hprt* T-cell assay is not suitable for the study of long term past exposure because of its poor long term memory. Four and one-half years after exposure the mutant frequencies of the exposed group were indistinguishable from the background frequencies of the Brazilian control group and mutant frequencies reported by others (Tates *et al.*, 1991; Branda *et al.*, 1993).

Our findings correspond with Natarajan *et al.*, (1991b) who reported estimations of the *hprt* mutant frequency and the frequency of chromosome aberrations of three subjects exposed to high doses of ionizing radiation during the radiological accident in Brazil, and five healthy non-exposed individuals from the same population. Estimates were carried out seven months after the original exposure by the autoradiographic assay. It was concluded that there was an elevated dose-dependent variant frequency (vF) in the lymphocytes of the exposed subjects. The mean of the natural logarithmic mutant frequencies reported was $5.4 + \ln 10^{-6}$ (2.2×10^{-4}). The increased mutant frequency persisted for several months. The mean of the natural logarithmic mutant frequencies reported for the unexposed individuals was $1.85 + \ln 10^{-6}$ (6.3×10^{-6}). Four out of five in the control group had mutant frequency determined by both the clonal assay and the autoradiographic assay and the results from the two assays were comparable.

An age-related increase in mutant frequency of 3.3% per year was found in our study. These data are in close agreement with a 3% per year increase in the *hprt* mutant frequencies in T-cells of five control populations which was reported by Tates *et al.* (1991). This relationship is statistically significant for the control group ($P=0.005$). However, it is obscured by the dose effect in the exposed group. As the dose effect diminishes with time, such significance becomes clearer in the exposed subjects as well. As the age distribution of the populations studied varies widely, so does the age-related increase in mutant frequency. It is therefore difficult to compare findings. Cole *et al.* (1988) reported an increase of 1.6% per year as opposed to $0.09/10^6$ cells per year of age described by Davies *et al.* (1992). In contrast, Strauss and Albertini (1979) and Bradley and Messing (1989) found no age-related increase in mutant frequency in their studies.

It is well established that cells containing aberrations slowly disappear from the circulating lymphocyte pool. It is likely that the mean half-life of PHA responsive T-cells varies

from individual to individual and to date there has not been agreement on the average value for humans. The differences found among these means favour the concept of lymphocyte heterogeneity. Just as there is a functional heterogeneity in the periphery, there is heterogeneity of intermitotic time (life span). Memory lymphocytes have a more rapid turnover in a way directly proportional to the commonness of the antigen they respond to. Lymphocytes with the longest life may be those committed to respond to rarely encountered antigens. This observation supports the supposition that long-lived memory is not maintained by long-lived T-lymphocytes (Buckton *et al.*, 1967; Michie *et al.*, 1992; Freitas and Rocha, 1993; Michie and McLean, 1993; Green *et al.*, 1995). T-lymphocyte lifespans vary from a few days to indefinite persistence and pools of peripheral lymphocytes seem to have differing rates of division depending on their type and their exposure to the antigen (Michie and McLean, 1993; Green *et al.*, 1995). Therefore, lymphocyte turnover will lead to an increase in mutant frequency with donor age even in the absence of selection for the TG^R phenotype (Green *et al.*, 1995). Estimates of such half-life have been reported as 1.45 years (Norman *et al.*, 1966), 3 years (Lloyd *et al.*, 1980), and approximately 4 years (Buckton *et al.*, 1967), based on the elimination of chromosome aberrations from the lymphocytes. Based on a 2.1-year half-life as determined in this study, the frequencies of the remaining cells present during the radiation exposure are 46.8%, 33.6%, and 22.6% in 1990, 1991, and 1992 respectively.

In our study, the mutant frequency decreases with time following exposure. Similarly, Aaron *et al.*, (1996) have reported on the loss of *hprt* mutations in monkeys over time following treatment of the animals with ENU and several other toxic compounds including cyclophosphamide, chloroethylmethanesulfonate, and adozelesin. They reported that ENU-induced mutant frequency increased significantly in a time-dependent fashion. The mutant

frequency yield peaked at approximately 80 days after treatment, followed by a reduction, and mutant frequency stabilized at an elevated level after approximately 150 days. No increase in mutant frequency was detected with all other compounds suggesting that the *hprt* assay is particularly insensitive to many mutagens. These studies demonstrated that the *hprt* assay has low sensitivity and, therefore, is of limited value for long term monitoring.

Among laboratories there is a considerable variability in reported mutant frequencies. Consequently, we emphasize the need for a large number of subjects per group in order to achieve an appropriate degree of confidence (Robinson *et al.*, 1994). This requirement can be difficult or impossible to satisfy with regards to human *in vivo* accidental exposure which is the particular case presented here. This is a variable over which researchers have no control and therefore will always be a very important confounding factor in the interpretation of human monitoring data. In addition, the life cycle of T-lymphocytes can also affect the overall mutant and mutation frequency in an individual. *Hprt* mutants are lost as a result of the natural turnover of the peripheral T-cells and there is evidence suggesting that there is also negative selection of the mutant cells (Albertini and DeMars, 1974; Strauss *et al.*, 1980; Curry *et al.*, 1995) in peripheral blood. Our results emphasize the fact that the *hprt* T-cell clonal assay is most effective for the monitoring of mutations when applied immediately following human exposure, giving only sufficient time for mutation fixation. However, this assay is of limited use following acute exposures that occurred many years earlier. In the case of ionizing radiation, continued chronic exposures maintain the pressure for mutation fixation making the *hprt* assay suitable for the monitoring of accumulated mutational effects.

CHAPTER IV. The Nature of Mutation in the Human *hprt* Gene Following *in vivo* Exposure to Ionizing Radiation of Cesium-137

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Abstract

The current study comprises the analysis of mutations in 10 individuals accidentally exposed to cesium-137 during the 1987 radiological accident in Goiânia, Brazil. Their exposures were among the highest experienced and range from 1 to 7 Gy. Peripheral T-lymphocyte samples were obtained 3.3 years after the original exposure, and mutation was studied at the *hprt* locus, using the 6-thioguanine selection assay. The mutational spectrum for the exposed population is comprised of 90 independent mutants. Based on T-cell receptor analysis, only 5% (5/95) were clonally related. Mutants were initially studied using RT-PCR and directly sequenced using an automated laser fluorescent DNA sequencer. Mutants that repeatedly failed to produce cDNAs were studied using a multiplex PCR assay and genomic DNA. Missense mutations were the most frequent event recovered, comprising 40% (23/57) of the spectral sample. An excess of events involving A:T base pairs was observed in this study, exhibiting significant difference (χ^2=12.7, p = 0.0004) when compared to the spontaneous spectrum. This finding may reflect the effect of ionizing radiation-induced damage, suggesting a potential similarity to prokaryotes. At the genomic level, 36.7% (33/90) of the mutants exhibited gross structural alterations as detected by multiplex PCR. Deletion events were over-represented in our spectral sample, accounting for a 2-fold increase when compared to the frequency observed in the spontaneous mutation database.

1. Introduction

In 1987 in Goiânia (Brazil) individuals were accidentally exposed to ionizing radiation after an abandoned radiotherapy unit was dismantled and sold as scrap, leading to one of the most serious radiological accidents to date. The source contained ^{137}CsCl (50.9 TBq or 1,375 Ci) which was partially dispersed over 2000 m^2, causing serious social, economical, and medical consequences, including four fatalities. Over 100,000 people were screened which revealed that more than 150 people had been exposed, both externally and internally, to doses greater than 0.1 Gy and as high as 7 Gy. Individual dose

estimates were derived by a combination of methods including internal dosimetry by both bioassays and whole body counting, cytogenetic dosimetry and external dosimetry (IAEA, 1988). Further, additional data on mutation at the glycophoryn A locus and chromosome translocation by *in situ* hybridization were in agreement with the original estimates (Straume *et al.*, 1991).

Ionizing radiation is a potent inducer of damage to DNA due to both free radical formation and direct DNA interactions (UNSCEAR, 1988). In aerated aqueous solutions, hydroxyl radicals are the most common reactive species produced by ionizing radiation. These radicals, as well as direct deposit of radiation energy onto DNA, can cause single- and double-strand DNA breaks, base modifications, apurinic/apyrimidinic sites, and protein-DNA cross-links (Huttermann *et al.*, 1978; Hutchinson, 1985; Teoule, 1987, Ward, 1988). The mutational consequences of ionizing radiation in mammalian cells have been studied in attempt to define its particular signature (Grosovsky *et al.*, 1988; Hakoda *et al.*, 1989; Fuscoe *et al.*, 1992a,b; Skandalis *et al.*, 1995). Understanding spectral samples and mutational specificity is essential to comprehend the mechanisms of radiation mutagenesis and the risk associated with radiation exposure.

Mutation spectra are usually considered to be mutagen-specific (Grosovsky *et al.*, 1988; Sarasin *et al.*, 1990). Different mutagens cause different kinds of lesions in the DNA, each with a unique mutagenic potency, DNA sequence preferences, and regional distribution of DNA damage. The spectrum also reflects the rate and accuracy with which the introduced lesion is repaired (Vrieling *et al.*, 1992). Thus, spectral analysis of mutations from an exposed population should provide valuable information about the origins of mutation and, potentially, the possibility of identifying specific exposures on the

basis of the type of mutations found.

The gene coding for the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) located at Xq26 (Pai *et al.*, 1980) is widely used as a marker for both *in vivo* germinal (Fujimori *et al.*, 1992) and somatic (Albertini *et al.*, 1993; Jansen *et al.*, 1994; da Cruz *et al.*, 1996) mutations as well as *in vitro* studies (O'Neill *et al.*, 1990a,b; Kent *et al.*, 1993; Deubel *et al.*, 1996). The T-lymphocyte clonal assay allows for the *ex vivo* selection of *hprt* mutants resistant to 6-thioguanine. In addition, this assay enables the estimation of clonal expansion, making it suitable for population monitoring (Albertini *et al.*, 1986; Moore *et al.*, 1993). The ability to collect mutants arising *in vivo* and their subsequent molecular characterization makes the *hprt* system an excellent choice to define mutational spectra in humans which is currently facilitated by both technical advances, including polymerase chain reactions (PCR), and extensive mutation database (Cariello *et al.*, 1992; Cariello, 1994).

PCR has been used by several laboratories to amplify the *hprt* gene and determine its DNA sequence alterations. Reports on *hprt* mutations include the *in vivo* (Recio *et al.*, 1990; Rossi *et al.*, 1990; Andersson *et al.*, 1992; Rossi *et al.*, 1992; Vrieling *et al.*, 1992; Hou *et al.*, 1993; Steingrimsdottir *et al.*, 1993) and *in vitro* (Giver *et al.*, 1993; Manjanatha *et al.*, 1994; Bao *et al.*, 1995) spectrum of spontaneous mutations in human T-lymphocytes. In addition, recent reports include spectra of mutations following cell treatment with mutagens (Walker and Skopek, 1993; Cariello *et al.*, 1994; Nelson *et al.*, 1994) and of human populations exposed to ionizing radiation (Shimahara *et al.*, 1995; Khaidakov *et al.*, *1996*).

The Brazilian radiological accident provided us with the opportunity to examine the mutational spectra of 10 individuals exposed to high doses (1 to 7 Gy) of ^{137}Cs ionizing radiation. The biostatistical information and mutation frequency data of our high-dose-spectral sample (HD-1991) was determined using mutants obtained from the second cohort in a longitudinal study of radiation-exposed individuals (da Cruz *et al.*, 1996) and are depicted in Table XVIIIa.

In this paper we expand the study of mutant frequency to include the HD-1991 mutational spectrum and comparison with a spontaneous spectrum (SS). Here the SS includes data from the human *hprt* mutant database (Cariello *et al.*, 1992; Cariello 1994), 40 individuals from a control group for A-bomb survivors (Shimahara *et al.*, 1995), and 31 healthy individuals from the Goiânia population (Skandalis *et al.*, 1997). We further compare our HD-1991 spectrum with a 41-mutant *hprt* mutational spectrum of individuals exposed to low doses (0.1-0.7 Gy) of ionizing radiation (LD-1990) in Goiânia (Skandalis *et al.*, 1997). The biostatistical information and *hprt* mutation frequencies of LD-1990 and Brazilian unexposed individuals can be seen in Table XVIIIb and c, respectively. An additional comparison was made between HD-1991 spectrum and the 48 mutants comprising the spectral sample of A-bomb survivors (AB) [Shimahara *et al.*, 1995].

2. Material and Methods

2.1. Donors

The study group comprises 9 males and 1 female exposed to 1-7 Gy of ionizing radiation (Table XVIIIA) during the ^{137}Cs radiological accident in Goiânia (Brazil) in September 1987. Individuals suffered both internal and external exposure. Blood samples

were collected in Brazil on a voluntary basis in 1991, 3.3 years after the accident.

Table XVIII. Biostatistical information and *hprt* mutant frequencies of individuals from the Goiânia population.

a. Individuals accidentally exposed to high doses of ionizing radiation. Blood collection of 1991[a].

Code #	Sex	Age	Dose (Gy)	PE (%)	MF/PE ($x10^{-6}$)
B126	M	36	1	28	21
B105	F	32	1	14	38
B81	M	17	1.3	26	10
B101	M	16	1.6	25	9
B135	M	50	2.1	44	9
B86	M	24	2.9	27	15
B103	M	16	2.9	25	12
B83	M	44	3	25	15
B162	M	46	4.4	13	28
B82	M	40	7	17	34
n		10			
Mean		32	3	24	19
SD_{n-1}		13	2	9	11
SE		4	0.6	3	3
95% CI		8	1	5	7

[a] From da Cruz *et al.* (1996).

b. Individuals accidentally exposed to low doses of ionizing radiation. Blood collection of 1990[b].

Code #	Sex	Age	Dose (Gy)	PE (%)	MF/PE ($x10^{-6}$)
B30	F	52	0.1	10	50
B27	M	29	0.2	2	90
B29	M	18	0.2	8	40
B28	F	29	0.4	1	810
B10	F	59	0.6	5	20
B26	F	9	0.7	5	10
B24	F	47	0.3	17	32
B37	F	29	0.3	19	7.5
B53	F	30	0.4	16	1
B39	M	47	0.5	10	3
B25	M	20	0.5	7	10
n		11			
Mean		33	1.6	10	126
SD_{n-1}		14	2.1	8	247
SE		3	0.5	2	60
95% CI		7	1.1	4	127

[b] From Skandalis *et al.* (1997).

c. Unexposed individuals. Blood collection of 1990[c].

Code #	Sex	Age	PE (%)	MF/PE ($x10^{-6}$)
BC2	F	21	18	10
BC4	F	42	31	20
BC5	M	50	26	14
BC7	M	ND*	7	17
BC8	F	35	23	15
BC10	M	20	12	11
BC33	F	4	29	4
BC66	F	41	16	33
BC67	F	35	21	15
n		9		
Mean		31	20	15
SD_{n-1}		15	8	8
SE		5	3	3
95% CI		12	6	6

[c] From Skandalis *et al.* (1997)
* Not determined.

2.2. Sample Collection

Peripheral blood was collected in 10 ml-Leucoprep tubes (Becton-Dickinson) and separated, following the manufacturer's directions. Separated MNCs were counted and diluted to 10^7 cells/ml in 50% calf serum (Professional Diagnostics), 10% DMSO (Sigma) and 40% RPMI 1640 (Hyclone). Cells were then frozen at approximately -1°C/min to -80°C after which they were stored under LN_2 and shipped to Canada where they were kept under LN_2 until required.

2.3. The *hprt* Assay

Published protocols for cell revival and the determination of mutant frequencies were followed (Curry *et al.*, 1993; da Cruz *et al.*, 1996). The 6-thioguanine-resistant T-cell clones were expanded to 1-3 x 10^7 cells for molecular analysis and cell pellets were stored at -20°C until required

2.4. Production of cDNA

A 2000-cell pellet was prepared from each mutant clone and cDNA was generated from total cell lysate in a 5µl reaction containing 2.5% of the non-ionic detergent NP40 (Sigma) to lyse the cells; 0.2 U/µl RNA guard (Pharmacia); 0.02 µg/µl BSA (Pharmacia); 0.01M DTT, 0.71mM dNTPs (Pharmacia); 0.1 OD_{260}/ml reverse primer II (Dalton Chemicals); 1x PCR Buffer (50mM Tris-HCl - pH 8.3; 75mM KCl; 3mM $MgCl_2$); and 5.7 U/µl Mo-MLV Reverse Transcriptase (Gibco/BRL). This step was done on ice. Samples were then incubated for 1 hour in a 37°C waterbath. Each unsuccessful amplification was repeated to a maximum of 6 times. The primer sets and their concentration were according to Yang *et al.* (1989).

2.5. cDNA Amplification

The amplification of *hprt* cDNA occurred in two stages of 30 cycles using nested primers in a Perkin-Elmer Cetus thermocycler (model 9600). The initial denaturation step was 5min at 95°C. After the cycling reaction steps were completed, a final extension step of 5min at72°C was run, followed by a storage step at 4°C. The primer sets and their concentration were according to Yang *et al.* (1989).

PCR I: The 5µl-RT product was used directly in a 50µl-reaction. The reaction mixture contained 1x PCR Buffer (15mM Tris-HCl - pH 8.5; 60mM KCl; 2.75 mM $MgCl_2$); 0.71mM dNTPs (Pharmacia); 5 U/µl *Taq* polymerase; 0.1 OD_{260}/ml forward primer I (Dalton Chemicals); and 0.1 OD_{260}/ml reverse primer II (Dalton Chemicals). Each cycle consisted of a denaturation for 20 seconds at 94°C, annealing for 30 seconds at 60°C and extension for 1min at 72°C.

PCR II: 1µl of the PCR I amplification product was used in 25µl-reaction. The reaction mixture contained 1x PCR Buffer (15mM Tris-HCl - pH 8.5; 60mM KCl; 2.75 mM $MgCl_2$); 0.71mM dNTPs (Pharmacia); 5 U/µl *Taq* polymerase; 0.1 OD_{260}/ml forward primer III (Dalton Chemicals); and 0.1 OD_{260}/ml reverse primer IV (Dalton Chemicals). Each cycle consisted of a denaturation for 20 seconds at 94°C, annealing for 30 seconds at 55°C and extension for 1min at 72°C.

After the second PCR round 1µl of gel-loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 30% glycerol in water) was added to 10µl of each reaction product and ran in 1% agarose gel, containing 2µl of ethidium bromide (10mg/ml) for verification of PCR success.

2.6. Multiplex PCR

Multiplex PCR, performed on genomic DNA as described (Gibbs *et al.*, 1990) was proven a rapid assay for the detection of *hprt* deletions. It involves the simultaneous amplification of the nine *hprt* exons using eight pairs of oligonucleotide primers in a single PCR.

2.7. DNA Extraction, Purification and Quantification

When DNA was successfully amplified in PCR II, the sample was reamplified using the original PCR I product as template. The reamplification produced sufficient DNA for direct sequencing. The DNA was precipitated by adding 10M ammonium acetate (0.2 volumes) and isopropanol (2 volumes). After gentle mixing, the mixture was left at room temperature for 15 minutes and then spun in a microcentrifuge (~14000 rpm) for 30 minutes. The supernatant was decanted and the pellet washed with 0.5ml of 70% ethanol

without mixing, followed by centrifugation for 15 minute. The supernatant was decanted and DNA pellet either dried at room temperature or dried in a Savant (Labconco), a speed vacuum drier. Finally the DNA pellet was resuspended in 25µl of sterile doubled destilled water overnight at room temperature. DNA concentration was measured fluorimetrically with a Hoefer TKO-102, and then stored at -20°C until sequenced.

2.8. Direct DNA Sequencing

The purified single-stranded PCR products were sequenced in an ALF DNA sequencer (Pharmacia) using "ReadyMIx Gel-ALF Grade" (Pharmacia), a 6% polyacrylamide sequencing gel, and prepared following the manufacturer's instructions. Sequencing was performed using the deoxy terminator cycle method and 50-100 ng of purified DNA as template. The sequencing reaction mixture contained 1x Buffer (30mM Tris-HCl - pH 9.0; 30mM KCl; 5mM $MgCl_2$); 5 U/µl *Taq* polymerase. Individual priming reactions contained primer concentration of 1.25 pmol and one of four ddNTP (Pharmacia) stop solutions (0.8mM ddATP; 0.4mM ddCTP; 0.08mM ddGTP; 0.8 ddTTP); and 60µM dNTP. Cycle sequencing involved preheating at 94°C for 4 minutes, followed by 25 cycles of denaturing (94°C-10sec), annealing (50°C-20sec), and extension (72°C-30sec). A final extension step of 5 minutes at 72°C was added to the protocol. The primer sets and their concentration were according to Yang *et al.*, 1989.

Upon completion, 6µl of loading dye (deionized formamide with dextran blue) was added to the products. Sequencing gels were run at 1500 V (38 mA), at 45°C, for 480 min. The sequence data was compared with the wild-type using SeqMan (DNAStar) software.

2.9. T-Cell Receptor Analysis

TCR assay was performed following de Boer *et al.* (1993). This method is based on analysis of restriction fragment length polymorphism of a PCR amplified rearranged T-cell receptor γ-gene using genomic DNA. If the TCR receptor failed using the primers for J1, a second amplification was done with primers for the J9.

2.10. Statistical Analysis

Mutational spectra were compared by class using the hypergeometric test via Monte Carlo techniques (Adams and Skopek, 1987) and chi-square test using Statistica (StatSoft) software.

3. Results

The T-cell clonal assay from 10 radiation exposed individuals yielded 221 mutants. Of these, 146 were successfully expanded and recovered for molecular analysis. cDNA was successfully generated by RT-PCR in 75% of the samples (110/146). Of these, 59 were randomly selected for sequencing. In each case the entire coding region of the *hprt* gene was sequenced using an automated laser fluorescent (ALF) DNA sequencer. Mutants for which cDNA was not produced were characterized by mPCR (Gibbs *et al.*, 1990). We also used the mPCR approach to characterize all potential exon losses from cDNAs.

3.1. T-Cell Receptor Analysis

As discussed by Nicklas *et al.* (1986, 1987, 1988, 1989) individual mutants recovered from the peripheral lymphocyte pool may either be the result of independent mutational events or of clonal expansion. Clonality can be determined by analyzing the

TCR in clones with identical mutations, as independent clones will display different rearrangements. Based on T-cell receptor analysis, only 5% (5/95) mutants were clonally related. The clonal distribution was 2 of the 59 mutant cDNAs and 3 of the 36 mutant genomic DNA. These duplicate mutants were removed from the spectral sample prior to statistical analysis. Therefore, a total of 57 independent cDNAs were sequenced and 33 independent genomic DNA were characterized by mPCR.

3.2. cDNA Alterations

The results for *hprt* cDNA mutations are summarized in Table XIX. The entire collection of independent cDNA alterations is given in Table XX.

Table XIX. *Hprt* cDNA mutational spectra by class: comparison of spontaneous and ionizing radiation exposed groups.

	Spontaneous		Radiation-induced mutations					
Mutation Class	SS[a]		AB[b]		LD-1990[c]		HD-1991[d]	
	Total	Freq.	Total	Freq.	Total	Freq.	Total	Freq.
G:C → A:T	35	15%	6	13%	5	12%	3	5%
A:T → G:C	11	5%	4	8%	3	7%	12	21%
Transitions	**46**	**19%**	**10**	**21%**	**8**	**19%**	**15**	**26%**
G:C → T:A	12	5%	3	6%	4	10%	2	4%
G:C → C:G	17	7%	3	6%	3	7%	0	0%
A:T → T:A	13	5%	4	8%	1	2%	3	5%
A:T → C:G	10	4%	3	6%	0	0%	3	5%
Transversions	**52**	**22%**	**13**	**27%**	**8**	**20%**	**8**	**14%**
Total Base Substitution	**98**	**41%**	**23**	**48%**	**16**	**39%**	**23**	**40%**
Exon Skipping	62	26%	9	19%	13	32%	12	21%
Frameshifts (-1)	19	8%	1	2%	1	2%	4	7%
Frameshifts (+1)	3	1%	1	2%	0	0%	3	5%
Complex	7	3%	0	0%	1	2%	2	4%
Tandem	2	1%	0	0%	0	0%	1	2%
Insertion/Duplication.	4	2%	0	0%	7	17%	2	4%
Seq. Deletion	43	18%	10	21%	3	7%	7	12%
Other	1	0%	4	8%	0	0%	3	5%
Total Mutants	**239**	**100%**	**48**	**100%**	**41**	**100%**	**57**	**100%**

[a] Spontaneous spectrum after Cariello (1994), Shimahara *et al.* (1995), and Skandalis *et al.* (1997); [b] A-bomb survivors study based on Shimahara *et al.* (1995); [c] Low dose exposed individuals during the radiological accident in Goiânia. Blood collection of 1990, after Skandalis *et al.* (1997); [d] This study includes the high dose exposed individuals during the Goiânia radiological accident. Blood collection obtained in 1991.

Table XX. cDNA sequence alterations identified at the *hprt* locus in 10 individuals accidentally exposed *in vivo* to high doses of ionizing radiation of ^{137}Cs.

Mutation Class	No. of mutants[a]	Mutant Code[b]	Base[c]	Exon	Sequence Context[d] and cDNA change	Codon[e]	Aminoacid Change	Comments
Base Substitution:								
1. Transitions:								
A → G	1	B126-7	370	4	ACT → GCT	124	Thr → Ala	
	2	B86-16; B105-3	598	8	AGG → GGG	200	Arg → Gly	
T → C	1	B101-7	107	2	TTT → TCT	36	Phe → Ser	
	5	B82-1; B83-5; B103-4; B126-9; B135-9	278	3	ATT → ACT	93	Ile → Thr	In 50% of the individuals
	1	B83-6	479	6	GTC → GCC	160	Val → Ala	
	2	B81-4; B162-5	548	8	ATT → ACT	183	Ile → Thr	
G → A	1	B103-8	101	3	AGG → AAG	34	Arg → Lys	
	1	B105-8	143	3	CGT → CAT	48	Arg → His	CpG site
	1	(B126-1; B126-4)	197	3	TGT → TAT	66	Cys → Tyr	
2. Transversions:								
A → C	1	B126-6	230	3	GAC → GCC	77	Asp → Ala	
	1	B83-9	593	8	TAC → TCC	198	Val → Ala	
T → G	1	B83-13	350	4	ATT → AGT	117	Ile → Ser	
A → T	1	B82-2	59	2	GAT → GTT	20	Asp → Val	
	1	B82-10	163	3	AAG → TAG	55	Lys → Stop	
	1	B86-18	498	7	AAA → AAT	166	Lys → Asn	
G → T	1	B86-17	489	7	TTG → TTT	163	Leu → Phe	
	1	B103-14	600	8	AGG → AGT	200	Arg → Ser	
Tandem	1	B101-1	124-125	2	ATT → GAT	42	Ile → Ser	

Table XX, cont.

Mutation Class	No. of mutants[a]	Mutant Code[b]	Base[c]	Exon	Sequence Context[d] and cDNA change	Codon[e]	Aminoacid Change	Comments
Complex:								
G → T	1	B101-2	489	7	TTG → TTT	163	Leu → Phe	Three RT-PCR products with identical base substitution and exons 2 & 3, and exon 3 loss.
G → A	1	B105-6	539	8	GGA → GAA	180	Gly → Glu	
			606	8	TTG → TTA	202	Leu → Leu	Silent mutation
Other								
G → C	1	(B126-2; B126-8)	234	3	CTG → CTC	78	Leu→Leu	Silent mutation
	1	B103-25	45-566	2-8	ACC<AGG...TGT>AGG			Normal multiplex PCR
	1	B162-13						No mutation found in cDNA
Frameshifts:								
1. Positive (+1):								
+C	1	B81-2	276(?)[f]	3	ATCCCATT	92	Creates premature stop codon (TAA)	
+G	1	B82-3	120(?)[f]	2	ATGGGACT	42/43	Creates premature stop codon (TGA)	
+G	1	B103-5	658(?)[f]	9	TAAGGATG	NA	First base pair in trailer region.	
2. Negative (-1):								
-A	1	B126-5	100(?)[f]	2	GGAAAGGG	33/34	Creates premature stop codon (TAA)	
-A	1	B135-10	383(?)[f]	4	GGAAAGAA	127/128	Creates premature stop codon (TGA)	
-G	1	B135-8	334(?)[f]	4	ACAGGGGACA	112/113	Creates premature stop codon (TAA)	
-G	1	B103-31	619	9	TTTGTGTCATT	207	Frameshift	

Table XX, cont.

Mutation Class	No. of mutants[a]	Mutant Code[b]	Base[c]	Exon	Sequence Context[d] and cDNA change	Comments
Exon Skipping[g]:						
	1	B135-2	28-134	2	Exon loss[h]	Normal multiplex PCR
	4	B83-15; B101-10; B103-7; B105-13	28-318	2-3	Exon loss[h]	Normal multiplex PCR
	3	B86-14; B103-2; B103-3	320-384	4	Exon loss[h]	Normal multiplex PCR
	2	B86-4; B105-9	385-402	5	Exon loss[h]	Normal multiplex PCR
	1	B103-27	403-485	6	Exon loss[h]	Normal multiplex PCR
	1	B83-11	486-532	7	Exon loss[h]	Normal multiplex PCR
Insert./Duplication:						
7 bp	1	B101-3	612-613	9	TCAT<ATAGCAT>GTTT	Inserted sequence located at I8:-4 to E9:3 creates new stop codon (TAG)
7 bp	1	B101-5	184-186	3	TCACA<CACACAG>TGTA	Original Sequence TCACA<T>TGTA; New sequence inserts Thr-His-Ser residues
Deletions:						
4 bp	1	B82-8	112-115	2	TTTATT<CCTC>ATGG	Creates premature stop codon (TAA)
6 bp	1	B105-7	226-231	3	CTTT<GCTGAC>CTGC	Removes Pro-Asp residues
7 bp	1	B101-4	592-598	8	GAA<TACTTCA>GGGA	Creates premature stop codon (TGA)
	1	B86-8	28-318	2-3	CGTG|<ATT...TGT>|AAT	Large deletion including I1 through I3
	1	B81-5	385-402	5	AAG|<AAT...GAA>|GAT	E5 exclusion caused by sequence deletion in I5
	1	B126-3	385-432	5-6	AAG|<AAT...CAG>ACT	E5 exclusion caused by deletion over I5 to E6:30
	1	B101-12	385-413	5-6	AAG|catctttggaaaacCACT	E5 exclusion with intronic insertion (I4:29174-I4:29187)
Total of mutants	57					

[a] Only independent mutants are shown;

[b] Clones in brackets shared the same rearranged TCR. Only one was considered for the spectral sample;

[c] Positions in coding region are numbered after Jolly *et al.*(1983) with adenine of the ATG initiation codon been the first nucleotide;

[d] Upper case indicates coding sequence while lower case intron sequence. Vertical lines show splice junctions and <> indicate breakpoints. Genomic *hprt* sequence and numbering after Edwards *et al.* (1990);

[e] First codon is the Methionine initiation codon (ATG) in the *hprt* coding region;

[f] As the affected base pair could not be precisely determined, we referred to the last base in the run as the one responsible to disrupt the reading frame;

[g] (Putative) splice mutant based on normal amplification of the nine *hprt* exons from genomic DNA; and

[h] represents exon exclusion from the *hprt* cDNA.

3.2.1. Base Substitutions: Missense mutations were the most frequent event in the spectrum of mutation of the *hprt* locus in this study. They comprise 40% (23/57) of the mutations in this database and were found in all exons, except 1, 5, and 9. The following classes of mutations were distinguished:

a. Transitions comprised 26% (15/57) of the spectrum and included 3 G:C → A:T and 12 A:T → G:C events. The most frequent recovered transition was an A:T → G:C event at position 278 (A<u>T</u>T → A<u>C</u>T) in the coding region. This event was recovered one time in 5 individuals. In order to exclude the possibility of a PCR artifact (e.g., the RT-reaction or cross contamination) we confirmed each mutation by sequencing the exon 3 from those individuals from genomic DNA. Only one of the G:C → A:T mutations occurred at a CpG site (position 143 - C<u>G</u>T → C<u>A</u>T).

b. Transversions comprised 14% (8/57) of the mutants and included 2 G:C → T:A, 3 A:T → T:A, and 3 A:T → C:G events. The G:C → C:G transversion was not recovered.

3.2.2. Frameshifts comprised 12% (7/57) of the mutations recovered. These included three "plus" and four "minus" frameshifts. The majority (6/7) occurred in a run of two or more bases (Table XX), although a single negative frameshift was recovered in a short repeat at position 207 (TTTGT<u>G</u>T).

3.2.3. Complex mutations involve two or more simultaneous changes. Mutant B101-2 produced three different bands on 1% agarose gel after RT-PCR. The largest product was a wild type size and had a base substitution at position 489 (G:C → T:A transversion) which is the fourth base pair in exon 7. The two smaller fragments both had the same base

substitution at position 489 as well as exon losses. The shortest fragment had lost exons 2 and 3 while the mid-sized fragment had lost exon 3. The nature of this mutation was also confirmed by mPCR. This G → T transversion event has been previously reported, though without the associated exon exclusions (Lukash *et al.*, 1991; Yang *et al.*, 1993). Mutant **B105-6** had two non-tandem point mutations within the coding region of the cDNA. They were both G:C → A:T transitions in exon 8, separated by 22 base pairs. One of these transitions was a silent mutation, affecting a leucine codon.

3.2.4. Tandem mutations: Only one tandem mutation was found among the radiation-exposed individuals. It involved a transition and a transversion at positions 124 and 125 (ATT → GAT).

3.2.5. Insertions and duplications have been grouped together. Mutant **B101-3** has a 7-base pair duplication in exon 9, between positions 612-613. The inserted sequence is GATTTGAATCAT>ATAGCAT<GTTTGTGTCATT and represents a repeat of the last 7 base pairs immediately before position 613. The first four bases in the inserted sequence are the last four bases in intron 8, and the last three bases of the inserted sequence represent the first three bases in exon 9. Mutant **B101-5** is a 7-base-pair duplication/insertion event in exon 3 as a result of the repeat of the sequence TCACA >CACACAG< TGTA. The duplication occurs between positions 184-186. The original sequence is TCACA<T>TGTA, where the highlighted T is lost and replaced by the entire duplication.

3.2.6. Deletions recovered in the cDNA were all short and in coding sequence. There are

three mutants with small deletions, **B82-8, B105-7** and **B101-4**, involving the loss of 4, 6, and 7 base pairs respectively, not surrounded by repetitive sequences. Four exon deletion events, involving deletion of one or more exons, were confirmed at the genomic level by mPCR and were included in the spectrum of cDNA alterations. Mutant **B81-5** cDNA had lost exon 5, though mPCR produced a shorter fragment. Genomic DNA sequencing of this fragment confirmed that exon 5 was present, but perhaps PCR primers were too close to the break points to accurately reveal any change of sequence. A scan of the region revealed two 6-bp sequence repeats 120 bp and 398 bp downstream from the 3'-end of exon 5. Slippage between these repeats would produce a 270-base pair deletion, resulting in a fragment consistent with the fragment recovered. Mutant **B86-8** was confirmed to be the consequence of genomic deletion spanning over exon 2 and 3. In mutant **B101-12**, exon 5 was lost along with the first 11 base pairs of exon 6. In addition, the insertion of a 13 base pair fragment from intron 4 was found at this site. The inserted sequence (Table XX) occurred between positions I4:29174 and I4:29187. Finally, mutant **B126-3** had exon 5 along with the first 30 base pairs in exon 6. Exon 5 was amplified by multiplex PCR, but not exon 6. This can be interpreted as a deletion within intron 6 with the 5' break point located at the 30^{th} base of exon 6.

3.2.7. Exon Skipping involves the precise loss of one or more exons at the cDNA level. Multiplex PCR, using genomic DNA, can distinguish exon exclusion from actual exon deletion events. However, we did not sequence the respective introns nor their splice junctions to characterize the splice mutants. Multiple bands after RT-PCR implicates splicing errors for the *hprt* gene. In this study, almost 14% (15 of 110) of the mutants had

multiple bands. This figure increases to about 18% if we included all exon loss events, e.g. product that had either multiple bands or smaller bands (20 of 110). These figures are not corrected for clonality. Several splice mutations (4/12) involved exons 2 and 3, 3 involved exon 4, 2 involved exon 5, and one each exon 2, 6, and 7 (Table XXI).

Table XXI. Summary of exon exclusion events from *hprt* cDNA mutational spectra, comparison of spontaneous and ionizing radiation exposed groups.

Exon	Spontaneous		Radiation-Induced					
	SS[a]		AB[b]		LD-1990[c]		HD-1991[d]	
	Total	Freq.	Total	Freq.	Total	Freq.	Total	Freq.
2	9	15%	0	0%	0	0%	1	8%
2 & 3	11	18%	1	11%	1	7%	4	33%
3	0	0%	1	11%	1	7%	0	0%
3 & 4	1	2%	0	0%	0	0%	0	0%
4	14	23%	1	11%	3	21%	3	25%
5	3	5%	1	11%	1	7%	2	17%
6	3	5%	1	11%	1	7%	1	8%
7	4	6%	1	11%	2	14%	1	8%
8	15	24%	3	33%	5	36%	0	0%
9	2	3%	0	0%	0	0%	0	0%
Total	62	100%	9	100%	14	100%	12	100%

[a] Spontaneous spectrum after Cariello (1994), and Shimahara *et al.* (1995), Skandalis *et al.* (1997); [b] A-bomb survivors study after Shimahara *et al.* (1995); [c] Low dose exposed individuals during the radiological accident in Goiânia. Blood collection of 1990, after Skandalis *et al.* (1997); [d] This study includes the high dose exposed individuals during the Goiânia radiological accident. Blood collection obtained in 1991.

3.2.8. "Other" mutations include mutants which failed to be classified in the categories discussed above. Mutants **B126-2** and **B126-8** had a silent mutation at position 234, due to a G:C → C:G transversion, involving a leucine codon. Identical codons have been used elsewhere in the *hprt* gene. Therefore, the 6TG-resistant phenotype cannot easily be associated with codon usage. Furthermore, this transversion was located in the core region of the active site of the HPRT protein, at a considerable distance from the splicing sites. In order to account for the 6TG-resistant phenotype, we suspect that a second mutation, possibly within one of the regulatory elements in the flanking regions of the *hprt* gene, may be involved. As this event was found twice in the same individual and both mutants

repeatedly failed to amplify the TCR region, we assume them to be clonally related. Mutant **B103-25** involved a 521-base pair deletion, spanning from position 45 through 566 in the coding sequence. Nevertheless, all the exons were amplified by multiplex PCR. This would be consistent with either inversion or translocation of a large fragment of the *hprt* gene which has not compromised the transcriptional regulatory elements and would explain the normal pattern of amplification of the nine exons by multiplex PCR. Finally, mutant **B162-13** did not exhibited any alteration in its cDNA.

3.3. Genomic Alterations

At the genomic level, 33 of the 90 mutants exhibited gross structural alterations as detected by multiplex PCR (Table XXII).

Table XXII. *Hprt* genomic DNA alterations identified by Multiplex PCR in 10 individuals accidentally exposed *in vivo* to high doses of ionizing radiation of ^{137}Cs.

Mutation Class	No. of mutants[a]	Mutant Code[b]	Exon	Frequency (%)
Deletions[c]:				
1. Total Deletion[d]	10	B82-9; B82-22; B83-10; B86-9; B103-10; B103-18; B103-19; B105-4; B105-5; (B135-4; B135-19)	1-9	30
2. Exon Deletion	5	B83-8; B103-1; B103-15; B135-11; B162-9	1	36
	1	B83-12	2-9	7
	1	B82-25	4	7
	2	B101-8; B103-36	7-8	14
	5	B101-9; B103-21; B103-32; B135-5; B135-7	9	36
Normal Multiplex PCR[e]	8	B82-17; B83-7; B86-13; B101-13; B103-30; B103-34; B105-2; B105-25	1-9	24
Other[f]	1	(B126-23; B126-30; B126-31)	3 and 9	3
Total of Mutants	33			100

[a] Only independent mutants are shown; [b] Clones in brackets shared the same TCR pattern. Only one was considered for the spectral sample; [c] Breakpoints were not determined; [d] TCR gene was co-amplified with *hprt* gene; [e] No cDNA was produced; [f] No genomic amplification of the two exons.

The genomic alterations are grouped as follows:

3.3.1. Sequence Deletions include mutants that failed to amplify one or more exons. The

deletion breakpoints were not determined. However, the genomic deletions were confirmed by a second multiplex PCR using both oligonucleotide primers for previously amplified exons together with oligonucleotide primers for the missing exon(s). A second failure to amplify missing exon(s) was the criterion to define exon deletion.

In this study, 14 of the 33 genomic mutants exhibited exon loss preferentially involving either the 5'- or the 3'-ends of the gene. Specifically, 72% of the exon deletion events affected either exon 1 (5/14) or exon 9 (5/14). The remaining deletions were represented by one exon 4 deletion, two exons 7 and 8 deletion and a large deletion spanning exon 2 through 9 **(B83-12).**

Twenty-seven percent (9/33) of mutants analyzed by multiplex PCR could not be classified as deletion events. One noteworthy mutant failed to amplify exons 3 and 9 using multiplex PCR. There were three such events **(B126-23, -30, -31)** in the same individual. Moreover, all three clones failed to amplify the TCR using both J1 and J9 PCR primers (primers sequence not shown) and likely represent a clonal run. The remaining 24% (8/33) produced no cDNA for analysis but produced a normal pattern after multiplex PCR amplification. We assume in these cases that the full mRNA is absent.

3.3.2. Total Deletions are identified by their failure to amplify any of the exons of the *hprt* gene. This subclass comprised 30% (10/33) of the mutation detected by multiplex PCR. As a positive control, the TCR gene from individual samples was co-amplified.

4. Discussion

A significant increase in *hprt* mutant frequency in human T-lymphocytes has been observed in individuals exposed *in vivo* to ionizing radiation (Messing and Bradley, 1985;

Seifert *et al.*, 1987; Hakoda *et al.*, 1988; Natarajan *et al.*, 1991b; Nakamura *et al.*, 1991; Seifert *et al.*, 1993; da Cruz *et al.*, 1996; Skandalis *et al.*, 1997), as well as *in vitro* studies (Sanderson and Morley, 1986; O'Neill *et al.*, 1990b). However, no difference in the nature of mutations recovered from exposed and non-exposed individuals has been reported (Shimahara *et al.*, 1995; Skandalis *et al.*, 1997).

In this report we chose to determine the spectral sample for the radiation-exposed individuals using PCR-based methodology over Southern Blot because of its resolution to detect point mutations. In addition, because there was an interim of 3.3 years from the time of the exposure to the time of sampling, gross alterations, mainly deletions, attributed to radiation exposure, would have impaired cell viability and could be lost from peripheral blood. In order to increase our chances of finding significant differences in the *hprt* mutation spectrum of the exposed group, we restricted the analysis to those individuals exposed to doses ranging from 1 to 7 Gy. To reduce possible individual donor differences on the type of mutations, only a small number of 6TG-resistant clones were analyzed per individual.

Using the hypergeometric test in 2 by 6 tables, we compared our HD-1991 spectrum of *hprt* mutations based on cDNA changes with the AB spectrum, LD-1990, and lastly with the SS spectral sample. We found no significant difference ($p \geq 0.05$). Furthermore, the inter-comparison of these groups showed no significant difference ($p \geq 0.05$). This did not change when both the high and low dose groups were pooled.

Comparing the HD-1991 spectrum with the SS revealed a significant increase in the frequency of A:T $\rightarrow$ G:C mutations in the exposed group ($\chi^2=17.72$, $p=0$). Interestingly, A:T $\rightarrow$ G:C transitions appeared to be enhanced by approximately 4-fold in

the population exposed to ionizing radiation. Peculiarly, A:T → G:C transitions at position 278 (5/57) were recovered in 5 of the exposed individuals (p=0.0002, Fisher exact test). Moreover, this change has not yet been reported in any of the spontaneous *hprt* data sets. Again we note that the base substitution at position 278 was confirmed at both cDNA and genomic levels. The elevated frequency of this specific event may be representative for past-exposure to ionizing radiation. We assume that T-lymphocytes from exposed subjects had survived the radiation hit with fixed permanent mutations that did not impair cell viability. This is not representative of recent exposure which leads to large deletions (Grosovsky *et al.*, 1988; Whaley and Little, 1990; Fuscoe *et al.*, 1992b; Hutchinson, 1995) which are considered the hallmark of radiation exposure. However, it is well established that exposure to ionizing radiation creates free radicals. The outcome of free radical damage could account for this mutation class.

It is noteworthy that A:T sites seem to be favored over G:C pairs as targets in our study population. The Chi-square test showed no statistically significant difference between the LD-1990 and AB groups. However, the occurrence of damaged A:T base pairs in our HD-1991 was significantly different (χ^2=12.7, p=0.0004) from the SS. Here A:T sites were involved in approximately 80% (18/23) of base substitutions as opposed to 35% (34/98) among the spontaneous mutants. This finding is consistent with experiments in bacteria (Glickman *et al.*, 1980; Levin *et al.*, 1982; Basu *et al.*, 1989; Wood *et al.*, 1990), bacteriophage (Conkling *et al.*, 1976; Wood *et al.*, 1990), and lower eukaryotes (Malling and de Serres, 1973), where detailed molecular analysis had been undertaken. Moreover, it has been reported that A:T base pairs are more susceptible to radiation and oxidation-induced mutagenic damage than G:C pairs in a test strain (TA102) of

Salmonella (Levin *et al.*, 1982). A similar sensitivity for A:T sites has been reported for ionizing radiation in *E. coli* (Basu *et al.*, 1989). These observations are consistent with the shift from G:C → A:T sites reported here.

All types of base substitutions at both AT and GC sites have been recovered after irradiation in prokaryotes and M13mp2 virus (McBride *et al.*, 1991). Similar changes have been reported in mammalian cells in a study using mouse lymphomas (Guerrero *et al.*, 1984). In addition, pyrimidine bases have been suggested to be more sensitive to ionizing radiation damage than purines, in the presence of O_2 (Breimer and Lindahl, 1985). Gamma radiolysis of pyrimidines in aqueous systems leads to the formation of several products including different hydroperoxide isomers, which decompose into more stable derivatives. Thymine bases are the most susceptible to modification, producing 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) as one of the major products (Teoule and Cadet, 1978). Several studies have shown that thymine glycol can stall DNA replication *in vitro* (Rouet and Essigmann, 1985; Clark and Beardsley, 1986) and when bypassed can produce a base substitution (Hayes and LeClerc, 1986). The radio-adduct $A^{8\text{-oxo}}$ (7-8-dihydro-8-oxoadenine) is another potentially mutagenic adduct which has been detected in DNA irradiated both *in vivo* and *in vitro* (Fuciarelli *et al.*, 1989; Malins and Haimanot, 1990). This premutagenic lesion also likely contributes to mutations that occur at A:T sites in the genome of prokaryotes and viruses following exposure to ionizing radiation (Glickman *et al.*, 1980). The $A^{8\text{-oxo}}$ has been implicated in single base pair deletions and possibly in A to C transversions (Wood *et al.*, 1992). However, the mispairing of $A^{8\text{-oxo}}$ with any base pair other than thymine would also result in a mutational event.

A 4-fold increase in the frequency of A:T → G:C transition on our exposed

population may thus reflect mutation due to a particular radiation-induced lesion, not unlike what is observed in prokaryotes. We suggest that the most likely mechanism would be thymine glycol mispairing with guanine. Whether this occurs during replication or reflects a repair defect cannot be determined but it must occur prior to the action of TG glycosylase (Brown *et al.*, 1985). Basu *et al.* (1989) reported that for the wobble mispair of thymine glycol and guanine to be mutagenic, it should preferably be 5' to a 3' purine. We noted that of the A:T sites recovered here, 75% (9/12) occurred 5' to a purine.

G → T transversions probably result from the radiation-induced lesion 7,8-dihydro-8-oxoguanine ($G^{8\text{-oxo}}$), a premutagenic adduct originally detected in gamma and X-irradiated DNA (Wood *et al.*, 1990). $G^{8\text{-oxo}}$ is thought to be removed by a DNA glycosylase, leaving an apurinic (AP) site in the DNA. AP sites can be mutagenic *in vivo*, where dATP is predominantly inserted opposite the lesion (Wood *et al.*, 1990). Consequently, G:C → A:T transitions may reflect the depurination of $G^{8\text{-oxo}}$ adduct. The deamination of a cytosine of a cytosine adduct would result in uracil or a modified uracil that would then pair with adenine during DNA replication, giving rise to a C → T transition (Hayes *et al.*, 1988). Cytosine deamination is a common source of spontaneous mutations and it is unlikely to be reflected in our spectrum as a radiation-induced event. G:C → C:G transversions were not recovered in our exposed population.

A single deletion spanning exons 2 and 3 was identified. One particular mutant had a deletion of exons 3 and 9 which may be best explained by an inversion within the *hprt* gene. The inversion would account for the disruption of control elements on the 5'-end of the gene, thus explaining the lack of transcriptional products. In addition, three small deletions involving repeat sequences were recovered and are hence consistent with the

slipped mispairing mechanism (Krawczak and Cooper, 1991).

The remaining deletions, including 10 total deletions of the *hprt* gene, account for 30% (27/90) of the mutations recovered from the exposed population. Such events are consistent with those recovered *in vitro* in the *aprt* gene in CHO cells following ionizing radiation exposure (Grosovsky *et al.*, 1988). These deletions share the common feature of not being readily explained by slippage events hence may reflect the direct effects of ionizing radiation-induced DNA strand breakage. This class of deletion is over-represented in our spectral sample by about 2-fold when compared to the frequency observed in the spontaneous database. Southern blot analysis of spontaneous *hprt* mutations in healthy individuals has shown that approximately 15% of those mutations are caused by gross structural alterations, namely deletions and insertions with break points occurring within and beyond the *hprt* locus (Albertini *et al.*, 1990b). The slightly elevated frequency of deletions seen 3.3 years after exposure may reflect T-lymphocyte turnover. We have previously (da Cruz *et al.*, 1996) calculated a half life of 2.1 years for T-cells carrying *hprt* mutations. Fuscoe *et al.* (1994) found about 7% of spontaneous *hprt* mutations are due to the total deletion of the gene. We found that the frequency of total deletion of the *hprt* gene in our exposed population is not significantly different from the expected frequency.

We note that among the genomic mutants from the radiation-exposed individuals 24% (8/33) lack mRNA. We assume that events within the promoter region are responsible, especially since this would also account for the normal multiplex PCR amplification.

One 6TG-resistant clone produced normal cDNA but without any detectable mutation in its coding sequence. Similar events have previously been reported in such cells exhibiting high levels of HPRT activity (Steingrimsdottir *et al.*, 1993). How this translates to TG-resistance remains unknown, although we did not measure the actual enzyme activity in this particular clone

Frameshift events were not significantly more common than found in the spontaneous database ($p \geq 0.05$). The majority of the frameshifts (6/7) occurred in a run of two or more bases, suggesting that these frameshifts were mediated by DNA sequence misalignments (Streisinger *et al.*, 1966). Each of the frameshifts produced in-frame stop codons terminating translation prematurely (Table XX).

The recovery of splice mutations in the spectral sample is similar in both the spontaneous and exposed groups ($p \geq 0.05$). Ionizing radiation does not seem to induce exon 8 exclusion, rather it seems to induce more exons 2 and 3 exclusion which may reflect induced V(D)J action.

Considering that the *hprt* gene has a large number of mutable positions (Jansen *et al.*, 1994) and that ionizing radiation deposits its energy in a relatively random fashion, mutational hot spots in the *hprt* locus would not be expected following exposure to radiation. In previously published studies, we reported a discrete, but significant increase in the mutant frequency of the exposed group (da Cruz *et al.*, 1996; Skandalis *et al.*, 1997). One could speculate that the increased mutant frequency was a result of physiological processes, such as clonality and T-cell turnover. However, the TCR analysis revealed that physiological processes were not an issue in our study, since there was only 5% clonality. As well, our sequencing data showed an increase in deletions not flanked by

repetitive sequences, as would be typical of spontaneous mutations. Thus, these events could reasonably be associated with true radiation-induced damage. In addition, the occurrence of damaged A:T sites was significantly different from the spontaneous spectrum which has been implicated as radiation-induced mutations. We suggest that perhaps the DNA damage seen in the exposed population was caused by free radical species originated by ionizing radiation at the time of exposure and, therefore, may account for the prevalence of mutations at A:T sites. It is rather difficult to assess what kind of free radical species would be implicated in such a mechanism because of the complex cellular environment exposed to ionizing radiation.

CHAPTER V. Somatic Microsatellite Instability in a Human Population Exposed to Ionizing Radiation of ^{137}Cs

Abstract

Tandem repeated DNA sequences, including microsatellites, are prone to somatic mutations that lead to changes in their length. Microsatellite instability (MIN) markers have become an important tool in the study of carcinogenesis and recently have been used to investigate gametic mutations in human populations exposed to ionizing radiation. In this study we determine the frequency of somatic MIN in 16 controls and 17 exposed individuals affected by the radiological accident in Goiânia (Brazil). A fluorescent PCR-based assay was performed for 6 markers (D6S135,f D8S135, D11S35, *Ankl*, *nm23-H1*, and *p53*). The PCR products were analyzed by polyacrylamide gel electrophoresis in an automated DNA sequencer. Data analysis was processed with SMART (Pharmacia) software. We examined a total of 200 and 190 alleles respectively and found that the putative microsatellite instability distribution in the two groups were not different($P \geq 0.05\%$). Our assay lacked sufficient sensitivity to discriminate between spontaneous and induced microsatellite instability. The *p53* marker was the most unstable, while the marker D11S35 exhibited no detectable instability.

1. Introduction

The human genome is comprised of approximately 10% of tandemly repeated DNA sequences. These sequences are generally associated with "noncoding" DNA and have no clearly ascribed functional attributes (Fowler *et al.*, 1988). Microsatellites are an abundant class of the tandemly repeated DNA sequences, composed of short runs of repeated motifs of fewer than 5-6 bp. It has been estimated that the human genome has about 100,000 microsatellites, which consist of $(A)_n$ and $(CA)_n$ repeats and closely related variations, including di, tri and tetranucleotide repeats. Microsatellites are highly polymorphic in the general population, but they are uniform in the DNA of an individual (Marra and Boland, 1995).

Microsatellites may be amplified by using PCR, in which primers are directed to unique sequences flanking the repeats (Weber and May, 1989). The tandemly repeated DNA sequences are prone to somatic mutations where base misalignment, insertions, and

deletions are more frequent than in other regions of the genome (Kunkel, 1990). In general, somatic mutations of microsatellites leads to changes in the length of the repeats, consequently changing the allele size. Microsatellite instability is a term, first proposed by Thibodeau *et al.* (1993), which refers to the changes in length of the microsatellites in the DNA extracted from neoplastic tissues as detected by shift in electrophoretic mobility.

Because the ubiquitous character and random distribution of microsatellites throughout the genome, as well as because the repeats are highly polymorphic, microsatellite markers have been used extensively for DNA fingerprinting, paternity testing (Fowler *et al.*, 1988; Chakraborty and Stivers, 1996) gene mapping and linkage analysis (Weber and May, 1989; Holmes, 1994), and distinguishing donor versus recipient cells following bone marrow transplantation (Oberkircher *et al.*, 1995). More recently, microsatellite markers have been used to identify specific mutations in inherited disorders including Huntington disease (Lesperance *et al.*, 1995), fragile X syndrome (Haataja *et al.*, 1994), myotonic dystrophy (Mulley *et al.*, 1991), and spinocerebellar ataxia type I (Shrimpton *et al.*, 1993). In addition, the instability of microsatellite markers has become a powerful indicator of repair proficiency (Parsons *et al.*, 1995) and has been demonstrated in neoplastic tissue, becoming an important tool in carcinogenesis (Cawkwell *et al.*, 1993; Thibodeau *et al.*, 1993; Peltomäki *et al.*, 1993; Honchel *et al.*, 1994; Mao *et al.*, 1994; Patel *et al.*, 1994; Wooster *et al.*, 1994; Cawkwell *et al.*, 1995; Eshleman and Markowitz, 1995; Negrini *et al.*, 1995; Tomlinson *et al.*, 1995; Eshleman *et al.*, 1996; Mao *et al.*, 1996). Moreover, germline mutation of human tandem-repetitive elements has been investigated in populations exposed to ionizing radiation (Kodaira *et al.*, 1995; Dubrova *et al.*, 1996).

Tumor development involves a multistage process in which oncogenes and tumor suppressor genes are involved, probably due to a progressive cascade of genetic events. Human malignancies are associated with somatic chromosome alterations, including translocations, deletions, and rearrangements (Solomon *et al.*, 1991; Cawkwell, *et al.*, 1993; Patel *et al.*, 1994). Recently, frequent somatic microsatellite instability has been reported in several tumors (see references above) and it was demonstrated that, in addition to microsatellites, housekeeping genes are also affected, as shown by a 100- to 1000-fold increase of *hprt* mutation frequencies (Bhattacharyya *et al.*, 1994; Eshleman *et al.*, 1996). Furthermore, microsatellite alterations were shown to be somatically acquired during cell division and then propagated as the cells underwent clonal expansion (Mao *et al.*, 1994).

Gene mutations, chromosome aberrations, cell transformation, and cell death are well known biological consequences of exposure to ionizing radiation. These consequences are caused by the deposition of radiation energy onto the DNA molecules, resulting in irreversible changes during DNA replication or as a consequence of error-prone enzymatic processing of lesions. It has been accepted that most DNA changes happen during the cell cycles immediately after exposure (Kronenberg, 1994; Kadhim *et al.*, 1995). Moreover, it has also been shown that exposure to ionizing radiation induces a persistent increase in accumulation of non-lethal heritable alterations in the progeny of irradiated cells (Fabre, 1983; Kronenberg, 1994; Kadhim *et al.*, 1995) and radiation-induced double-strand breaks are an important trigger of genomic instability (Jeggo and Kemp, 1983). Thus, ionizing radiation may induce a transmissible microsatellite instability that could be detected after many cell cycles in the progeny of irradiated cells. To date, studies of radiation-induced instability have used either rodent cells, rodent-human hybrid

cells, or immortal human cell lines (Kronenberg, 1994). In addition, *in vivo* analysis of somatic microsatellite instability, induced by ionizing, radiation has not yet been reported.

The radiological accident in Goiânia (Brazil) provided us with a singular opportunity to investigate the potential induction of microsatellite instability *in vivo* in a population exposed to ionizing radiation of ^{137}Cs. On September 13, 1987, a radiotherapy unit, containing 50.9 TBq (1,375 Ci) of radioactive $^{137}CsCl$, was removed from its protective housing and subsequently ruptured for scrap. As a result, 249 individuals were accidentally exposed to ionizing radiation. Over 150 people incurred doses ranging from 0.1 to 7.0 Gy, including some internal exposure due to skin absorption and ingestion of ^{137}Cs. Individual exposure ultimately lead to progressive acute radiation syndrome, along with severe skin lesions, and at last four deaths. Dosimetry for internal contamination was estimated using whole body count and monitoring of feces and urine (IAEA, 1988). External exposure was estimated using biological indicators (Ramalho *et al.*, 1988; Ramalho *et al.*, 1991). The glycophoryn A locus and chromosomal translocation by *in situ* hybridization (FISH) were later used for biodosimetry of some of the exposed individuals and were in agreement with the previous estimates (Staume *et al.*, 1991).

The present study examines the frequency of microsatellite instability in peripheral lymphocytes in 16 control and 17 exposed individuals from the Goiânia population. The principal hypothesis was that if there was an increased instability in the exposed group this could be differentiated from the frequency observed in the control group, using fluorescent PCR-based assays for six microsatellite markers/loci. The PCR products were analyzed by polyacrylamide gel electrophoresis in an automated DNA sequencer.

2. Material and Methods

2.1. Samples

Peripheral blood was collected in 1990 using 10ml-Leucoprep (Becton-Dickinson) tubes following the manufacturer's instructions. MNCs were frozen at -1°C/min above the liquid phase of LN_2 in 50% calf serum (Professional Diagnostics), 10% DMSO (Sigma), and 40% RPMI 1640 (Hyclone). Cells were stored under LN_2 and shipped from Brazil to Canada on dry ice. Samples were collected on a voluntary basis from both non-exposed and exposed individuals and their biostatistical information can be found in Tables XXIII and XXIV, respectively.

Table XXIII. Biostatistical information for the Brazilian non-exposed individuals comprising the control group.

Donor	Sex	Age (years)	Donor	Sex	Age (years)
BC96	M	19	BC116	F	33
BC97	F	25	BC119	F	37
BC98	M	18	BC123	F	9
BC102	F	36	BC124	F	10
BC107	F	42	BC125	M	6
BC112	F	42	BC127	M	24
BC113	M	25	BC130	M	28
BC115	F	43	BC139	M	26

Table XXIV. Biostatistical information for the Brazilian exposed group.

Donor	Sex	Age (years)	Dose (Gy)	Donor	Sex	Age (years)	Dose (Gy)
B82	M	40	7.0	B91	F	17	0.4
B83	M	44	3.0	B92	F	34	0.2
B84	F	48	0.3	B93	F	10	0.1
B85	F	60	0.6	B95	M	48	0.5
B86	M	24	2.9	B100	M	9	0.2
B87	M	29	0.15	B103	M	16	2.9
B88	F	38	0.3	B126	M	36	1.0
B89	F	30	0.45	B137	M	10	0.6
B90	F	26	0.2				

2.2. DNA Extraction

Cell samples were lysed and genomic DNA was extracted using Wizard Kit for genomic DNA purification (Fisher/Promega) following the manufacturer's instructions. DNA pellet either dried at room temperature or dried in a Savant (Labconco), a speed vacuum drier. Finally the DNA pellet was resuspended in 25µl of sterile doubled destilled water overnight at room temperature. DNA concentration was measured fluorimetrically with a Hoefer TKO-102, and then stored at -20°C until required.

2.3. Primers

In this study 6 microsatellite markers were used. Our rationale for marker selection was based on previously reported association of individual markers to specific solid tumors typically found in a population comparable to our study population, including the chromosomes most likely to be involved in radiation-induced malignancies. The PCR product size range expected was from 98-162 bp (Table XXV).

Table XXV. Microsatellite markers location and tumor types potentially associated with instability.

Marker/ Locus	Fragment Size (bp)	Repeat	Location	Reference	Rationale
D11S35	152-162	$(GT)_{17}$	11q22	Litt *et al.*, 1991b	Ovary, colon, skin, uterine cervix, and breast cancer
D6S105	116-138	$(CA)_{23}$	6p	Weber *et al.*, 1991	MHC complex
D8S135	152-162	$(GT)_n$	8p	Wood and Schertzer, 1991	Hematological malignancies, colorectal tumors, malignant melanoma, ovary, breast, and prostate cancer
ANK1	107-113	$(AC)_{14}$	8p11-21.1	Polymeropoulos *et al.*, 1991	DNA repair and replication, breast cancer
nm23-H1	98-104	$(CA)_n$	17q21.3	Hall *et al.*, 1992	Metastasis suppressor gene, breast cancer
p53	103-135	$(CA)_n$	17p	Jones *et al.*, 1992	Tumor suppressor gene, breast cancer

2.4. Fluorescent PCR

The PCR mixture contained 1x PCR buffer (10 mM Tris-HCl - pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1 mM TMAC); 125 µM each dNTP (Pharmacia); 2.5 U/µl *Taq* polymerase; and 50 pmol forward and 50 pmol reverse primers (Dalton Chemicals) in a 50 µl-reaction. PCR reactions were performed individually for each microsatellite marker, using 50-100 ng of genomic DNA. TMCA (tetramethylammonium chloride) was added to the PCR buffer to improve the stringency of the hybridization reactions and, therefore, enhance PCR specificity. TMAC was shown to eliminate non-specific amplification without any inhibitory effects on *Taq* polymerase (Hung *et al.*, 1990).

Our thermal protocol also included a touchdown approach which was used to reduce non-specific priming. Our approach was to decrease 1°C in the annealing temperature in each cycle, starting from 65°C until it reached 55°C. The last 20 cycles were performed at 55°C. Each cycle of annealing temperature lasted 1 minute. The overall thermal protocol can be seen in Table XXVI.

Table XXVI. The optimized thermal protocol used in this study includes the touchdown approach to amplify microsatellite markers using fluorescent PCR methodology.

	Temperature	Time	No. of Cycles
Initial Denaturation	94°C	5 min	1
Cycling Denaturation	94°C	1 min	(1 cycle/temp) 20
(Touchdown) Annealing	(65-56)-55°C	1 min	
Cycling Extension	72°C	1 min	
Final Extension	72°C	5 min	1
Hold	4°C	forever	N/A

2.5. Polyacrylamide Gel Electrophoresis and Data Analysis

The fluorescent single-stranded PCR products were separated using an ALF DNA sequencer (Pharmacia) using "ReadyMIx Gel-ALF Grade" (Pharmacia), a 6%

polyacrylamide denaturing gel, prepared following the manufacturer's instructions. The final PCR products were diluted in a ratio of 1:3 in aqueous solution containing loading dye (deionized formamide with dextran blue). Polyacrylamide gels were run at 1500 V (38 mA), at 45°C, for 400 min. A fluorescent sizer (Pharmacia) comprised of 10 known DNA fragments ranging in size from 50-500bp (10 fmol of each fragment/μl) was included in each gel. This sizer was used as a reference allowing for fragment size estimation.

ALF2SMA® (SMART, Pharmacia) was used to calculate the height and area of each fluorescent product. Excel 7.0 (Microsoft) was used to generate the electropherograms.

2.6. Assessment of Microsatellite Instability

Samples which consistently exhibited novel allele peaks for a particular marker were classed as having putative instability at that marker. In this study, every novel allele classified as MIN fell within the range of stutter activity. Because we lack the practical comparison between two different tissues from the same donor, we established criteria to assign instability to a particular microsatellite marker. The criteria are as follow: 1) we considered only the proportion of samples that were informative (i.e., heterozygous); 2) a new allele must exhibit a size difference of as little as one base pair and a peak area larger than any of the stutter peaks; and 3) as PCR amplification frequently produces spurious bands, each sample was assayed at least twice to confirm the result.

2.7. Statistical Analysis

All the P values were calculated performing the χ^2 test, using Statistica (StatSoft).

3. Results

Tables XXVII and XXVIII summarize the results of microsatellite fragment analysis for the present study. The proportion of samples that were informative (i.e. heterozygous) with the six microsatellite markers was 86.3% and 82.3% for non-exposed and exposed groups, respectively. We examined a total of 200 and 190 alleles respectively and found that the microsatellite instability distribution in the two study groups was not statically different ($P \geq 0.05$). When all six markers were considered together, the somatic instability for non-exposed and exposed groups were 5.3% and 4.5%, respectively. The *p53* microsatellite marker was found the most unstable, accounting for 4.2% of all informative markers. The least unstable marker was D11S35 which did not exhibit detectable instability in our study.

Table XXVII. Putative microsatellite instability results from 16 unexposed individuals from the Goiânia population.

Marker	n	No. of alleles	Heterozygous	Homozygous	MIN
D6S105	16	32	16	0	3
D8S135	15	30	11	4	2
D11S35	16	32	13	3	0
ANK1	16	32	13	3	1
nm23-H1	16	32	14	2	0
p53	16	32	15	1	4
Total	95	190	82	13	10

Table XXVIII. Putative microsatellite instability results from 17 individuals exposed to ionizing radiation during the Goiânia radiological accident.

Marker	n	No. of alleles	Heterozygous	Homozygous	MIN
D6S105	17	34	15	2	1
D8S135	16	32	12	4	2
D11S35	17	34	11	6	0
ANK1	17	34	14	3	1
nm23-H1	17	34	16	1	2
p53	16	32	16	0	3
Total	103	200	84	16	9

The electropherograms on Figure 8A-F show one case for each microsatellite markers that does not exhibit instability, while the electropherograms on Figure 9A-E show additional peaks considered as microsatellite instability in this study. Non informative samples were easily determined, as only one fluorescent peak was seen for those samples (Figure 9 F).

Stutter bands were generally present and are assumed to be caused by the *Taq* polymerase in the PCR failing to read through the repeat region and therefore generating spurious fragments (Litt, 1991; Cawkwell *et al.*, 1993 and 1995). In our study, the presence of such additional peaks exhibited a constant pattern for each marker when the PCR amplifications were carried out under similar conditions. Additionally, we observed that the constancy was reproducible when different individuals were assayed for the same marker under similar conditions (data not shown).

4. Discussion

The use of fluorescent PCR and the use of the ALF DNA sequencer (Pharmacia), combined with the ALF2SMA® analysis proved to be a rapid and efficient protocol which improved the assessment of microsatellite instability. The initial cost of similar equipment can be very high, however, as a multifunctional system is a valuable tool, costs associated with fluorescent PCR analyses can be relatively low. In addition, the automated system does not impose the nuisance of radioisotope uses, the unavoidable need for specialized facilities and personnel, as well as vigorous waste management policies.

A number of studies using tumor and non-tumor tissues have been carried out. However, to date only a single study has reported instability of microsatellites in normal

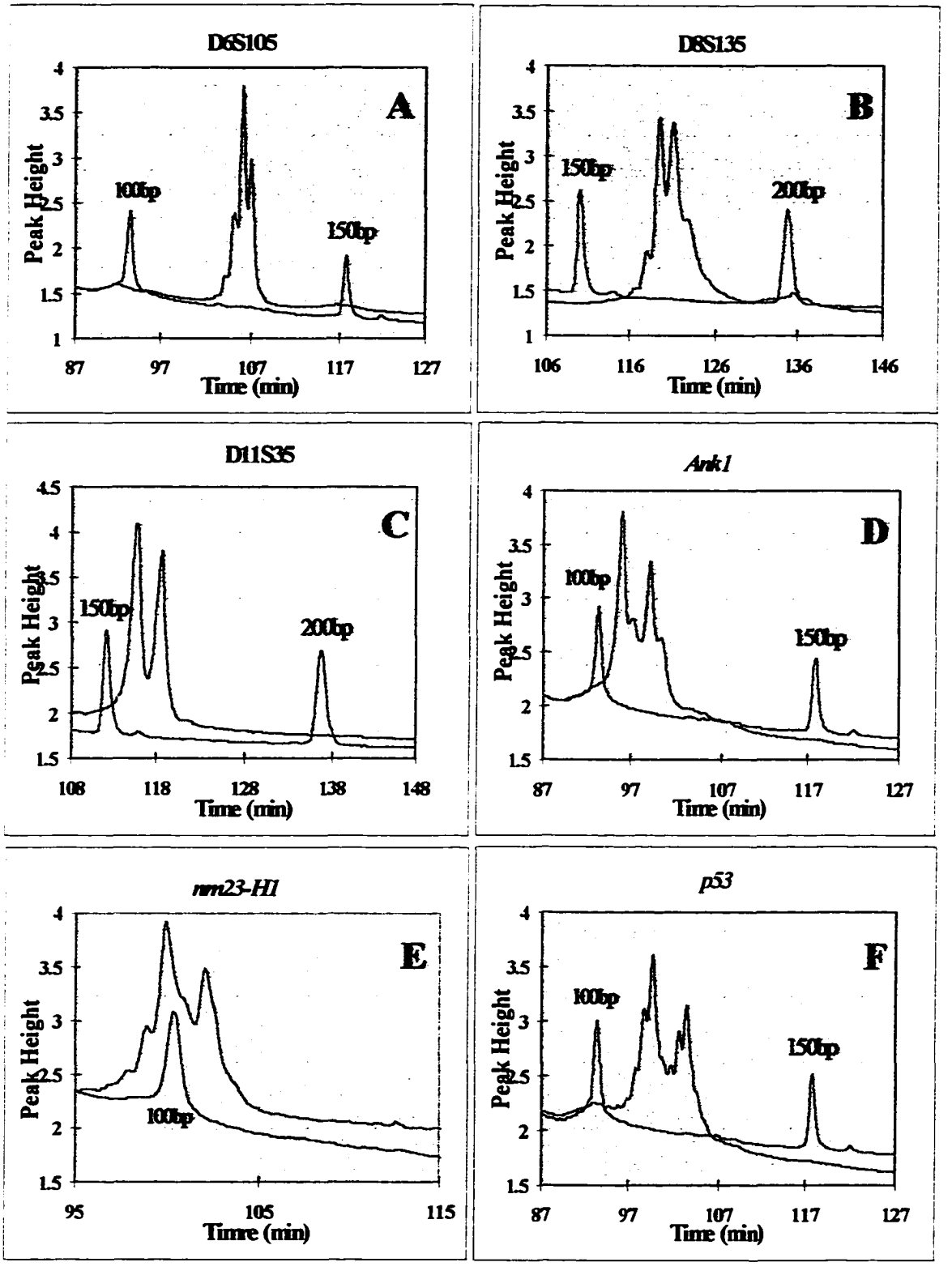

Figure 8. The electropherograms depict normal peak patterns of the microsatellite markers used (green). (A) D6S105; (B) D8S135; (C) D11S35; (D) *Ank1*; (E) *nm23-H1*; and (F) *p53*. Red peaks represent known fragment sizes used as a ladder for fragment size estimation. Peak height is in unit of retention.

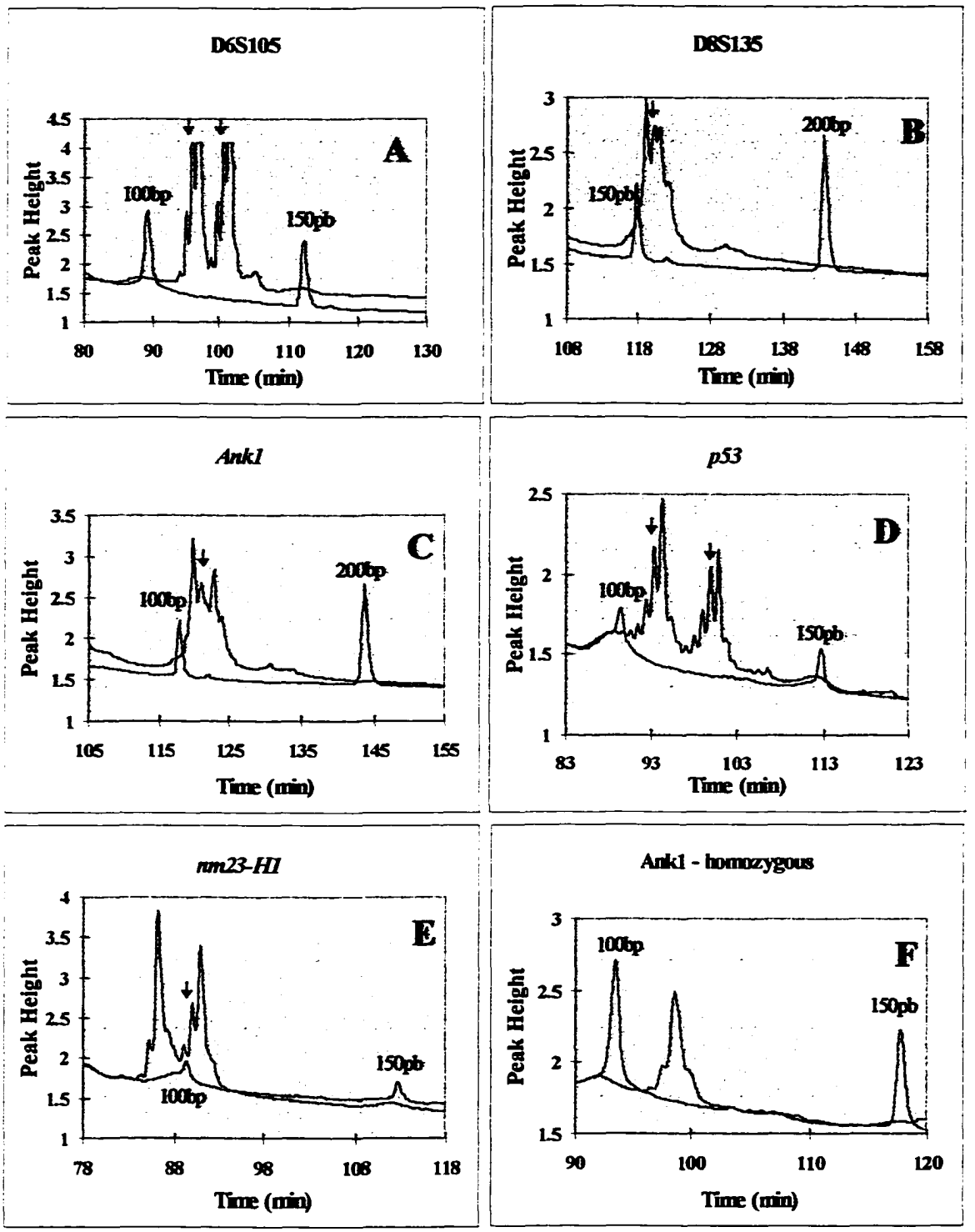

Figure 9. The electropherograms depict microsatellite alterations (green) , indicated by the presence of additional allele. (A) D6S105; (B) D8S135; (C) *Ank1*; (D) *p53;* (E) *nm23-H1*; and (F) non-informative samples as determined by only one fluorescent peak. Red peaks represent known fragment sizes used as a ladder for fragment size estimation. Arrows indicate the extra alleles. Peak height is in unit of retention.

tissue, using T-cell clones. This suggests that normal human genome may be remarkably stable, making somatic instability uncommon in the normal population (Kronenberg, 1994; Wooster *et al.*, 1994; Eshleman and Markowitz, 1995). The lack of evidence of microsatellite changes in normal cell populations is probably due to a lack of sensitivity in the assays. If a rare cell in a population developed microsatellite alterations, the new allele would not be detected amid the large background of normal cells. However, a recent study using clonally related cells demonstrated that phenotypically normal tissues could harbor microsatellite instability, which is consistent with our findings (Hackman *et al.*, 1995; Parsons *et al.*, 1995). In addition, a study similar to our current one has been carried out in our laboratory using the same six microsatellite markers to investigate potential instability in AT probands which have been implicated in genetic instability (Meyn, 1995). It has been found that approximately 41% of the informative alleles in AT homozygotes demonstrated potential microsatellite instability (Steele *et al.*, in preparation). This is approximately a 3.5-fold increase when compared to our results. We found 5.3% and 4.5% of putative microsatellite alterations in our study populations of non-exposed and exposed individuals, suggesting that mismatch repair deficiency (poor proficiency) is compatible with cell development.

We chose MNC because this cell fraction contained a large number of T-lymphocytes which undergo nuclear divisions while in circulation. We reasoned that T-cell clonal expansion would increase our chances of finding those rare cells with poor repair proficiency, thereby increasing the chance to demonstrate microsatellite alterations in our populations. The changes in allele size would have arisen from misalignment during the DNA replication. Moreover, somatic microsatellite alterations have been shown to be

acquired during division in a single transformed cell and propagated as this cell underwent clonal expansion (Mao *et al.*, 1994).

We did not find gel to gel, lane to lane, and PCR to PCR variation to be a problem as the results were consistent for repeated assays under similar conditions. Nevertheless, we could not discriminate between exposed and non-exposed individuals with respect to induction of microsatellite instability by ionizing radiation. The informative samples gave similar allele ratio results with each primer set for both the exposed and non-exposed groups and no statistically significant difference was found between the exposed and non-exposed groups. However, the process of induced instability may require multiple genetic alterations subsequent to radiation exposure. It has been hypothesized that in order to accumulate multiple mutations, potential cancer cells need to disable some of the cellular mechanisms that normally safeguard the fidelity of the cell's DNA (Loeb, 1991).

Most human cancers, including those induced by radiation exposures, involve the accumulation of multiple mutations, and most do not appear until later in life. Genetic predisposition may, however, accelerate the tumorigenic process (Kronenberg, 1994).

The potential use of somatic microsatellite instability arising spontaneously in the normal population could become an effective tool in identifying individuals with somatic mutations in DNA repair genes. However, our current approach lacks the sensitivity to discriminate between spontaneous and ionizing radiation-induced microsatellite alterations and it is, therefore, not suitable for population monitoring. It is possible that radiation exposure *in vivo* may yield microsatellite alterations, but at frequencies below detection. A number of variables must be investigated prior to understanding the mechanism of formation and the kinetics behind the microsatellite instability. Nevertheless, we assert that

the identification of clonal microsatellite alterations in body fluid have important implications in cancer detection and diagnosis.

CHAPTER VI. Radiation Risk Estimation in Human Populations: Lessons from the Radiological Accident in Brazil

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Abstract

The development of radiological and nuclear technologies and the deployment of nuclear weapons have made ionizing radiation one of the most studied human mutagens. Exposure to ionizing radiation produces DNA damage which can result in mutation and cancer, making the risk associated with human exposure a critical issue. In this paper we estimate the risk associated with radiation exposure for individuals exposed to ^{137}Cs during the 1987 Goiânia radiological accident. Using combined regression slopes from both the *in vivo hprt* mutant frequency and micronucleus frequency data we estimated a doubling dose of 173 (± 47) cGy for these two endpoints. This is in close agreement with the published estimates for low dose rate and chronic exposure to low-LET radiation. We obtained risk estimates of about 24-fold increase in dominant disorders in the post-exposure generation of the directly exposed population. No detectable increase was found in the population at large. The risk of carcinogenesis in the directly exposed population was found to be increased by a factor in the range of 1.4 to 1.5. The small sample size in this study requires a large element of caution with respect to risk estimates interpretation. Moreover, the doubling dose estimates prepared here are derived from lymphocytes. This somatic data may require additional considerations for both cancer and certainly germ-line events. Nevertheless, the risk of carcinogenesis and genetic harm for this population are good indicators of the potential genetic damage imposed by ionizing radiation to the Goiânia population.

1. Introduction

Ionizing radiation is perhaps the most extensively studied human carcinogen. Whether this reflects the potential benefits associated with the use of radiological and nuclear technologies or the fear associated with the deployment of nuclear weapons (Land, 1980) is not certain. What is clear is that exposure to ionizing radiation can damage DNA and result in both mutation and cancer. While natural radiation may induce mutation and hence contribute to genetic variation (Pochin, 1980a,b; Denniston, 1982), the vast majority of the biological effects of radiation are viewed as harmful. It is thus the estimation of the risk associated with exposure

to ionizing radiation that is the subject of this paper.

The carcinogenic effects and genetic risks of radiation have been extensively detailed in the literature (see BEIR III, 1980; UNSCEAR 1982 and 1988, and references therein) and are, in a general sense, reasonably well understood. H.J. Muller (1927) first demonstrated the mutagenic potential of ionizing radiation in *Drosophila* and pointed this out as a potential genetic hazard. Since then, several laboratories and research groups have worked together to define and determine the risk imposed by exposure to ionizing radiation (Denniston, 1982; ICPEM, 1983; Sankaranarayanan, 1993, UNSCEAR, 1988 and references therein). Considerable amounts of data have been obtained on both rodent germ cell mutations (Ehling, 1991, UNSCEAR, 1988 and references therein) and human somatic mutations (Albertini, 1994 and references therein).

The dose-response relationship, especially at low doses however, remains unclear. The questions of whether there is a linear or exponential dose response and whether there is a discrete threshold below which biological consequences are uncommon, remain unanswered. Similarly, we are uncertain of the extent to which such factors as LET (low energy transfer) and dose rate affect the outcome of exposure. Essentially, the extent of human illness arising from an increase in mutation rate due to exposure to ionizing radiation remains a critical issue (ICPEMC, 1983; UNSCEAR, 1988; Sankaranarayanan, 1993; de Serres, 1994).

This report attempts to estimate the doubling dose of ionizing radiation using somatic mutation obtained from individuals exposed *in vivo* to ^{137}Cs gamma radiation during the radiological accident in Goiânia (Brazil). We also endeavoured to ascertain the genetic implications of radiation exposure in this defined population. The impact that such exposure might have on the health and welfare of the exposed individuals and the general population was

determined by estimating the relative genetic risk and the risk of carcinogenesis in that population. All the risk estimates performed in this work follow the standard protocols of UNSCEAR (1988). We recognize that the small sample size requires a large element of caution when interpreting the results of this analysis, which should be considered as an empirical exercise.

2. The Goiânia Radiological Accident and the Somatic Mutations Database

The data considered in this report are drawn from the Brazilian population group exposed to ^{137}Cs during the 1987 Goiânia radiological accident in September 1987. An abandoned radiotherapy unit containing 93g of $^{137}CsCl$ was removed from its protective housing by two individuals, who thought that the lead assembly could have some scrap value and who were unaware of the fact that it contained 50.9 TBq (1375 Ci) of radioactive material. During the two weeks after the rupture of the radiotherapy unit, several people were exposed both externally and internally to ionizing radiation to doses as high as 7 Gy.

As reported by IAEA (1988), the exposed individuals incurred initial acute whole body external exposures followed by chronic whole body exposure at low dose rates from internally deposited ^{137}Cs. The lack of precise information on exposure histories further obscured the dose estimations. However, most of the individuals were exposed over a protracted period at varying dose rates with exposure ranging from acute to chronic. Doses were estimated by means of 1) biological dosimetry using chromosome aberration analysis; 2) internal dosimetry by measuring the radioactivity in urine and faecal samples, as well as whole body counting; and 3) external dosimetry based on the reconstruction of the accident and on the basis of the previously known radiological properties of ^{137}Cs. Using the method of "null" mutation at the

glycophoryn A locus and chromosome translocations by *in situ* hybridization, Straume *et al.*, (1991) obtained biodosimetric results one year after exposure that concurred with chromosomal aberration results obtained immediately after the accident.

Our study examined *in vivo* mutation levels using two genetic endpoints, the X-linked *hprt* gene (Saddi *et al.*, 1996; da Cruz *et al.*, 1996; Skandalis *et al.*, 1997) and micronucleus frequencies (da Cruz *et al.*, 1994) in both exposed and control populations. Summaries of our data, including sex and age distribution are given in Tables XXIX and XXX.

Table XXIX. Summary of the mutant frequency and the induced rate of mutation of individuals exposed to ^{137}Cs using the *hprt* locus of T-lymphocytes as the endpoint.

	Mean lnMF ($+\ln 10^{-6}$) (SD)	Mean Dose (cGy) (SD)	Sample Size	Mean Age (years) (SD)	Sex Distribution	Induced mutation/cGy
Exposed Group	2.54 (1.69)	92.4 (135)	36	26.2 (14.7)	41.7% (female)	1.15×10^{-2}
Control Group	1.48 (1.5)	Unexposed	21	23.6 (14.3)	52.4% (female)	

Table XXX. Summary of the micronucleus frequency and their induced rate in peripheral T-lymphocytes of individuals exposed to ^{137}Cs and controls.

	Mean MN Frequency (SD)	Mean Dose (cGy) (SD)	Sample Size	Mean Age (years) (SD)	Sex Distribution	Induced mutation/cGy
Exposed Group	1.5×10^{-2} (1.0×10^{-2})	77.6 (13.4)	75	24.1 (16.2)	50.7% (female)	6.6×10^{-5}
Control Group	9.9×10^{-3} (6.6×10^{-3})	Unexposed	65	32.7 (16.1)	69.7% (female)	

3. Risk Estimation and Extrapolation for the Brazilian Data

3.1. Estimation of Carcinogenesis for the Goiânia Population

Risk estimates for hereditary harm and carcinogenesis are based on a linear dose-response relationship. This is consistent with our data from the radiation-induced mutation kinetics observed in this population. Figure 10 shows the curve-fitting analysis of the mutant

frequencies in the *hprt* locus versus absorbed radiation dose. Figure 11 depicts the same relationship for micronucleus frequency of the Brazilian exposed and control groups.

Radiation can cause chromosome aberration and mutations. However, the same events do occur spontaneously. Therefore it follows that the consequences of radiation exposure are not novel, rather we expect an increase in frequency of the spontaneous damage. This can lead to cancer induction, one of the most important somatic effects of low-dose radiation exposure (BEIR III, 1980). There is no current method available to identify those individuals who are likely to develop specific disorders such as cancer due to radiation exposure. Even if an exposed individual later develops cancer, it is not yet possible to prove that radiation exposure was the underlying cause. However, the existence of radiation-induced cancer can be defined on the basis of statistical excess above the natural incidence.

We propose to estimate the relative risk and the incidence (number of new cases) of carcinogenesis in the Goiânia exposed population compared to the population at large. Relative risk is simply the ratio of the number of cases observed in the exposed population to the number of the cases expected in the control population (UNSCEAR, 1988). IAEA (1988) reported that 249 individuals were exposed during the 1987 radiological accident. Throughout the 8 years since exposure, 6 cases of malignant tumours and 2 benign tumours have arisen in the exposed population alone (Table XXXI).

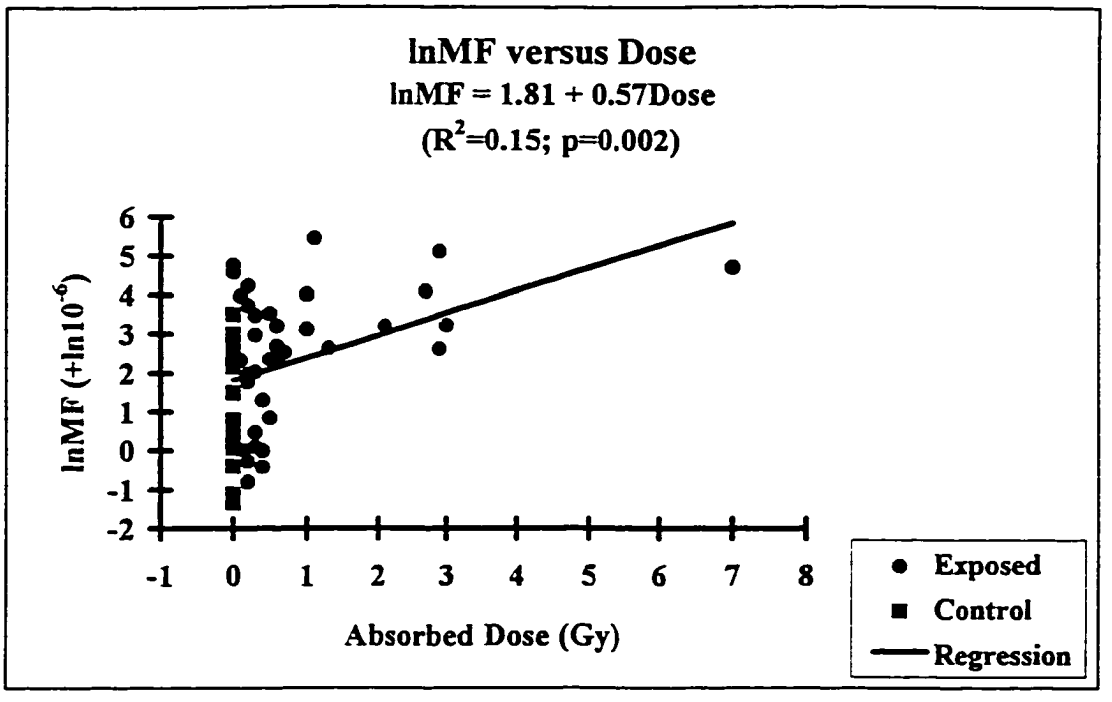

Figure 10. Dose response curve for the mutant frequencies at the *hprt* locus versus absorbed radiation dose in people accidentally exposed to ^{137}Cs and control subjects from Goiânia (Brazil).

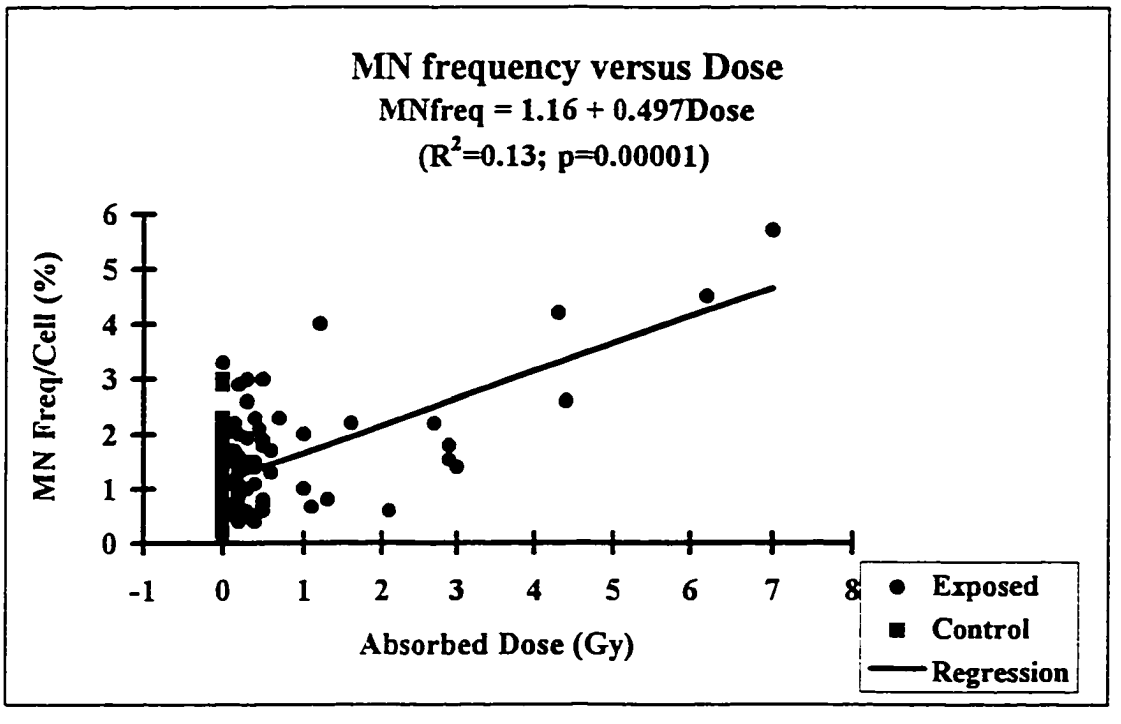

Figure 11. Dose response curve for the micronucleus frequencies in peripheral T-lymphocytes versus dose in individuals accidentally exposed to ^{137}Cs in Goiânia (Brazil) and control group.

Table XXXI. All cases of tumours which arose in the population exposed to ionizing radiation of ^{137}Cs during the radiological accident in Goiânia (Brazil). Data collected between 1988 and 1995.

Patient	Sex	Dose (Gy)	Age[a] (years)	Tumour Type	Tumour Location	Histopathology/Comments
1	M	0.2	21	Skin benign lesion	NR[b]	NR
2	F	ND[c]	59	Skin cancer	Face	NR
3	F	0.4	58	Benign lesion	Ovary	Serous papillary multilocus cystoadenoma
4	F	0.2	31	Carcinoma	Breast	Infiltrating ductal carcinoma with pulmonary, cerebral, and orbital metastasis
5	M	ND	50	Melanoma	NR	Disseminating metastatic melanoma
6	M	ND	20	Carcinoma	Rhinopharynx	Tumour and metastatic cells had integrated EBV genome
7	M	7	36	Carcinoma	Oesophagus Prostate	Squamous cell carcinoma Adenocarcinoma

[a] Approximate age at exposure.
[b] Not reported
[c] Not determined

In order to avoid overestimation of carcinogenesis, our assessment did not consider the two benign tumours nor the two cases of skin cancer as these types of lesions were not included in the baseline data (ACCG-IPB, 1995) from the Goiânia population. Therefore the incidence of cancer in the exposed population alone was 0.016 (1.6%) over the eight years elapsed since the accident. Although our non-age-adjusted baseline data spans only 6 years (1988-1993), epidemiological data show no significant changes in pattern over these years (ACCG-IPB, 1995). Therefore, for practical purposes we assume the extrapolation of these data to 1995 as a reasonably valid approach. The mean incidence of cancer over the six years (1988-1993) was 1.1% in the Goiânia population at large. This value is significantly smaller than that found in our exposed population, which expressed a value of 1.6%. The ratio of these two values reflect a 1.5-fold increase in cancer incidence in the exposed population compared to the population at large. We did not have a control population to compare with our cohort of 249 individuals. To overcome this obstacle we derived the expected cases of cancer in a population of similar size,

based on the baseline incidence of cancer in the Goiânia population at large. This gave us a value of 2.7 cases in 249 individuals. The exposed population had 4 cases, therefore the relative risk is 1.4. The two methods are mutually consistent which lead us to believe this is a reasonable estimation of risk.

3.2. The Direct Method

Risk is described by Edwards (1986) as a "chance of injury." The genetic risk estimation induced by radiation to human populations is based on extrapolation from animal experiments. As low-LET radiation-induced mutations are stochastic events, the probability of the event depends upon dose (Ehling, 1991). Thus, the prediction of risk with a large degree of confidence is possible only when describing the behaviour of large populations.

The extrapolation procedure we have used to estimate the genetic risk in humans is known as the "direct method" (Ehling, 1966). This approach is based on experimental data from the mouse and is derived from Ehling's pioneering work (Kratochvilova, 1979; Denniston, 1982; Ehling *et al.*, 1982). This method estimates the total risk of dominant genetic disease to the first generation progeny of an exposed human population. Using this procedure the estimated rates are extrapolated from mouse skeleton or cataract data (Denniston, 1982; Ehling, 1988; Sankaranarayanan, 1993).

In this report the extrapolation of risk estimation is based on autosomal dominant and X-linked diseases. The dominant cataract mutation data was chosen to determine the genetic risk for the Goiânia population using the direct method and can be seen in Table XXXII. One significant advantage of using the dominant cataract data is that these data can be used directly for the estimation of the genetic damage expressed in the first generation of human populations

(Ehling *et al.*, 1982).

Table XXXII. Estimation of the genetic risk in the offspring of the Brazil population after exposure to ^{137}Cs during the Goiânia radiological accident.

Estimation by	Dominant Cataract Mutation	References
Induced MF in mouse (mutation/gamete/Gy)	$0.45 - 0.55 \times 10^{-4}$	Ehling, 1991
Multiplication Factor	47	McKusick, 1988
Total Dose Commitment (manSv)		
1. Exposed Population[a]	3.5×10^5	IAEA, 1988
2. General Population[b]	1.4×10^3	
Mutational Component	1.0	UNSCEAR (1988)
Reproductive Factor	0.4	UNSCEAR (1988)
Total of Estimated Dominant Mutation:		
1. Exposed Population	$296 - 362/10^6$ live birth	
2. General Population	$1.2 - 1.5/10^6$ live birth	
Total of Expected Dominant Mutation:	$10 - 20/10^6$ live birth	UNSCEAR (1988)
Relative Risk:		
1. Exposed Population	~ 24	
2. General Population	no detectable increase	

[a] based on 129 individuals who exhibited both internal and external contamination (IAEA, 1988).
[b] based on the exposure to public in the streets of Goiânia (IAEA, 1988).

The main assumption of this method is the sensitivity of the selected mouse genes to induced dominant mutations is representative for human genes at which dominant mutations can arise spontaneously (Denniston, 1982; Ehling *et al.*, 1982; Ehling, 1988; Selby, 1990; Ehling, 1991; Favor, 1993; Sankaranarayanan, 1993 and 1994).

The following equation was used to predict the risk of dominant mutations in the offspring of the Brazilian population after the Goiânia radiological accident:

$$ExpD_{Human} = IndMF_{Mice} \times MF_{Mice\ to\ Human} \times TDC_{Human} \times MC \times RF$$

where:

$\mathbf{ExpD_{Human}}$ is the expected number of induced dominant mutations in humans; $\mathbf{IndMF_{Mice}}$ is the induced mutation frequency in mice (mutation/gamete/Gy, $0.45\text{-}0.55 \times 10^{-4}$, following Ehling (1991)); $\mathbf{MF_{Mice\ to\ Human}}$ is the multiplication factor for the overall dominant mutation frequency in humans. It is used to convert the induced mutation rate of dominant cataracts to the

estimation of the overall dominant mutation rate (Ehling *et al.*, 1982; Ehling, 1991). This factor is the ratio of all dominant mutations (1,443) to dominant cataracts (31) according to McKusick (1988). $\mathbf{TDC}_{\text{Human}}$ is the total dose commitment to the Goiânia population from ^{137}Cs from the radiological accident (IAEA, 1988). **MC** reflects the total impact of newly created mutations in the exposed populations and is assumed by UNSCEAR (1982) as being one (1) for autosomal dominant and X-linked conditions; **RF** is the reproductive factor as suggested by UNSCEAR (1988). It represents F/L, F being the main reproductive age (about 30 years) and L being life expectancy at birth (about 75 years).

In order to calculate the risk estimates for our study population, we determined the total dose commitment following Edwards (1986) and Brøgger (1993). The total dose commitment to the Goiânia population at large was estimated as been 1.43×10^3 manSv. This estimate is the result of the mean doses on the streets of Goiânia multiplied by the number of individuals in that population (1.43×10^{-3} Sv $\times 10^6$ people - IAEA, 1988). The total dose commitment to the directly exposed individuals was estimated to be 45.15 manSv (3.5×10^{-1} Sv $\times$ 129 people - IAEA, 1988). We then extrapolated this dose estimate to one million people, therefore the directly exposed population has a total dose commitment equivalent to 3.5×10^5 if 10^6 people were exposed in similar conditions.

Using the direct method we estimate the relative risk to the directly exposed population of Goiânia to be about 24-fold higher than the expected dominant mutations anticipated to the population at large. No detectable increase was found in the population at large. To date, 16 healthy children have been born to those exposed to doses ranging from 0.2 to 1.6 Gy including a history of both external and internal exposure. This figure includes infants that were exposed *in uteros*, 3 to 6 months into gestation. *Hprt* mutant frequencies were assayed in these four

children and no relationship to dose was determined (Saddi *et al.*, in press). To date, abortions, stillbirth, and malformations have not been reported within the directly exposed population. This corresponds with both BEIR III (1980) and UNSCEAR (1988) conclusions that there is no direct evidence of genetic effects of ionizing radiation in humans, despite the overwhelming evidence in animals. Our observations are also in agreement with observations on the children of atomic bomb survivors in Hiroshima and Nagasaki. No statistically significant increase was observed in the exposed group in any of the parameters studied (such as congenital malformations, stillbirth, death among live-born children up to 26 years of age, physical development, sex ratio, chromosome aberrations, etc.) [Schull *et al.*, 1981; Neel *et al.*, 1989 and 1990].

3.3. The Doubling Dose

The doubling dose (DD) is defined as the dose necessary to induce as many mutations as occur spontaneously in one generation (i.e., twice background) [Ehling, 1991; Favor, 1993]. The DD estimate is a good indicator for the evaluation of the potential human hazard for a given exposure (Neel *et al.*, 1990).

The Goiânia study populations exhibited sufficient overlap to allow combined additive analysis of the genetic endpoints, after Neel *et al.* (1990). We have used this approach based upon the additively combined regression analysis of the *hprt* mutant frequency and the micronucleus frequency in peripheral T-lymphocytes of both exposed and control populations to estimate the genetic doubling dose of radiation exposure in that population.

The outlined approach uses the ratio of the combined slope (Table XXXIII) over the mutational component, which reflects the total impact of newly created mutations in the

exposed populations. The mutational component (MC) is the ratio of the mean induced mutation frequency over the mean background mutant frequency. In this study the combined mutational component represents the mean of the *hprt* and micronucleus mutation frequencies (Tables XXIX and XXX). The mean induced mutation frequency is the difference between the overall induced mutant frequency for exposed subjects and the mutant frequency of the control group.

Table XXXIII. Summary of the regression variables for the two somatic endpoint indicators of radiation exposure in the Goiânia population.

Somatic Endpoint	Regression (± SE)
hprt Mutant Frequency	0.574 (± 0.181)
Micronucleus Frequency	0.497 (± 0.109)
Combined	1.071 (± 0.29)

For the study population, the combined mutational component is 0.62 (62%). The somatic doubling dose calculated in this manner is 173 (± 47) cGy. This value is close to the currently accepted doubling dose of 100 cGy (UNSCEAR, 1982 and 1988), and is within the range of 50-250 cGy suggested by the BEIR III (1980) for low dose rate or chronic exposures to sparsely ionizing radiation, such as X- or γ-rays, calculated primarily using mouse data.

4. Conclusions

Determining the genetic hazards of exposure to ionizing radiation is difficult. Auerbach (1975) concluded that the estimation "of risk is a dangerous procedure, because it will create the impression that our conclusions are meaningful, whereas in reality they are so full of uncertainties as to be practically meaningless." This was again reiterated by Crow (1981) in a lecture entitled "How well can we assess genetic risk? Not very."

We have attempted to use our experimental data to obtain some sense of the risk

involved in human exposures. While this has provided a useful lesson, the small size of the exposed Goiânia population makes the interpretation of these results difficult. This is further complicated by the nature of the assumptions and the limited data upon which estimates are made. Nevertheless, they can be considered as a reasonable indicator as is currently possible of the level of genetic damage for that population.

The added risk of carcinogenesis is maybe genuine. This is consistent with the number of individuals in the exposed population who had developed cancer by 1995. However, as it is rarely possible to categorically attribute a specific cancer to ionizing radiation, we can only conclude that it appear that the probability of carcinogenesis is indeed higher in the exposed population.

Our estimates of risk appear small, but whether these represent a "significant" risk must be answered at the individual level. Unexpectedly high risk estimates based on studies of populations exposed to low-dose radiation can not be rejected out of hand (Land, 1980). However, in terms of decision making, factors other than risk may play a role. Nevertheless, some knowledge of the size of the risks is important for an intelligent and responsible comparison of these alternatives (Pochin, 1980a,b).

Keeping in mind that risk estimation encompasses complex calculations and is based upon a number of uncertainties and assumptions, we support Sobels' observations (Sobels, 1989 and 1990) that human somatic mutation could be used as a good indicator of ionizing radiation to humans. Therefore extrapolations to the gametic level would be possible if the relationship between somatic and gametic mutations in mouse and humans were better understood. This would require the assumption that mice and humans are affected the same way. At this time however, we can not state this unequivocally.

A better understanding of the exposure-risk question could be enhanced by a comparative analysis of the human and the mouse database at both somatic and gametic levels. Such an analysis is still required. Similarly, there is a need for improved experimental methods designed for human model systems. These additional data will improve our concept of risk estimation, and ultimately offer better protection to the genetic pool of future generations.

CHAPTER VII. Overall Discussion

Exposure to ionizing radiation is a fact as old as life itself, however, the perception of the potential danger of radiation has become a common worry of the modern world. Humans and all other living creatures have been constantly exposed to natural sources of radiation, including cosmic radiation and external and internal irradiation from radionucleides within the organism. Moreover, the development of radiological and nuclear technologies has, to some extent, contributed to human exposure due to these man-made sources. Over the past century, few nuclear issues have commanded as much public and scientific attention as those related to radiation. Public awareness of the risks associated with radiation has increased, perhaps in response to the contribution from man-made radiation. Nevertheless, there is still a gap between scientific documentation and public perception of exposure to radiation

Prevention, detection, assessment, and risk estimation are important procedures to evaluate the impact that occupational, accidental, therapeutic, or environmental radiation exposures may impose on human health. A clear understanding of both biological and physical consequences of such exposure provides the bases for critical decisions concerning both the handling of radioactive material and the development of monitoring and follow-up approaches. The first line of defense is prevention, including both occupational and accidental exposures. Prevention could be readily achieved by using physical methods to monitor involved personnel and by reinforcing the safety guidelines and the laws relating to the management of radioactive material.

Scientific knowledge relating to radiation effects is continuously expanding and advances in modern biology should make it possible to determine the sensitivity to ionizing radiation of an individual person. This would contribute to minimizing individual exposure to medical radiation, identifying those individuals who require increased clinical surveillance for cancer, and finally to determining whether radiation is indeed the cause of some cancers and some genetic disorder.

Harm to the environment and ultimately to humans caused by radiation is almost entirely associated with nuclear accidents or the use of nuclear weapons. Such unfortunate conditions provide unique opportunities to investigate the potential biological effects of radiation exposure on human health. The nuclear weapons legacy comprises the dropping of two atomic bombs in August 1945 on Hiroshima and Nagasaki. Approximately 600,000 inhabitants of those two cities were affected by this terrible historical episode. Of that number only about 100,000 individuals survived. The Hiroshima and Nagasaki residents comprise the largest group of individuals who have been followed medically for over 40 years. The list of nuclear accidents has been increasing. Two major accidents world-wide were the Chernobyl accident in April 1986 which was the largest nuclear accident in the Eastern world, and the Goiânia radiological accident in September 1987 which was the largest nuclear accident in the Western world. Both events were characterized by communication gaps between the population, political decision-makers, media, and medical experts.

The Goiânia radiological accident provided us the opportunity to understand some of the biological effects of exposure to ionizing radiation imposed in a population. The exposed people have expressed great concern about their future health, including

particular anxiety regarding the potential for the genetic harm in their offspring and their own risk of cancer development. From 1990 until the present we have been monitoring the genetic health of some of the individuals exposed to ionizing radiation in the Goiânia accident, with doses ranging from 0.1 to 7 Gy. Our approach was first to investigate and determine possible exposure by using the micronucleus assay as the cytogenetic endpoint. Secondly, we carried out studies at the molecular level, using the *hprt* gene as the molecular endpoint. We conducted a longitudinal study to determine both the level and the fate of *hprt* mutation frequency in T-lymphocytes of individuals exposed to high doses of ^{137}Cs as well as the nature of mutations induced in that population. We then performed a comparative study with the spontaneous spectrum of mutations at the *hprt* gene, as well as comparing our spectrum of mutations to both the Goiânia low dose group spectrum and the A-bomb survivor spectrum. Again, at the molecular level we have investigated the possible effect of ionizing radiation on the induction of microsatellite alterations using MNCs from exposed and non-exposed individuals. Finally, we used both the micronucleus and the *hprt* data to estimate the genetic harm and the risk of carcinogenesis in the Goiânia population.

Using the micronucleus assay we performed an investigative study on a random sample of 276 individuals. Even 1 year after exposure we have shown the assay to have strong predictive utility for high dose exposures as long as comparison to control individuals is used. Unfortunately, the micronucleus assay cannot detect low levels (<0.2 Gy) of ionizing radiation. Additionally, prediction of individual dosage (true dosimetry) is not possible. We conclude, however, that the micronucleus assay is a useful biological dosimeter for human populations even if blood samples are taken a year after exposure to

ionizing radiation. While the human lymphocyte micronucleus assay cannot replace detailed chromosomal analysis for precise estimations of radiation doses, it can be used as a quick predictive model of exposure for screening purposes.

When we performed the longitudinal study on the 10 individuals exposed to high levels of ionizing radiation with doses ranging from 1 to 7 Gy, two major findings were revealed: 1) The *hprt* mutant frequency was higher in those exposed to high doses of ^{137}Cs ionizing radiation during the Goiânia radiological accident than in the control group obtained from the same population; 2) The *hprt* mutant frequency of the exposed individuals decreased gradually over time, showing the *hprt* T-cell assay is not suitable for the study of long term past exposure because of its poor long term memory. Four and one-half years after exposure the mutant frequencies of the exposed group were indistinguishable from the background frequencies of the Brazilian control group and mutant frequencies reported by others (Tates *et al.*, 1991; Branda *et al.*, 1993). In addition, we found an age-related increase in mutant frequency of 3.3% per year which is consistent with a 3% per year increase in the *hprt* mutant frequencies in T-cells of five control populations (Tates *et al.*, 1991). We also estimated a 2.1-year half-life for those T-cells having *hprt* mutations, which is directly responsible for the decrease in mutant frequency with time, following exposure. This study demonstrated that the *hprt* assay has low sensitivity and, therefore, is of limited value for long term monitoring.

In order to establish possible differences between our spectral sample comprised of mutants obtained from 10 individuals exposed to high doses of ionizing radiation, we used the hypergeometric test in 2 by 6 tables. We compared our HD-1991 spectrum of *hprt* mutations based on cDNA changes with the AB spectrum, LD-1990, and lastly with the SS spectral sample, and found no significant difference ($p \geq 0.05$). However, Chi-square

analysis, comparing our HD-1991 spectrum and the SS, revealed that there was a potential difference between these two groups centered on base substitutions. A significant increase of about 4-fold in the frequency of A:T → G:C mutations was found in the exposed group. Peculiarly, A:T → G:C transitions at position 278 (5/57) were recovered in 5 of the exposed individuals. This particular transition has not yet been reported in any of the spontaneous *hprt* data sets and may even be representative of past-exposure to ionizing radiation. It is noteworthy that A:T sites seem to be favored over G:C pairs as targets, in our study population, a trend which was shown to be statistically significant. Damaged A:T base pairs in our HD-1991 comprised about 80% (18/23) of base substitutions as opposed to 35% (34/98) among the spontaneous mutants. This increase in the frequency of A:T → G:C transition on our exposed population may reflect mutation due to a particular radiation-induced lesion, most likely due to mispairing of radiation-induced thymine glycol with a guanine. This finding is consistent with experiments in bacteria (Glickman *et al.*, 1980; Levin *et al.*, 1982; Basu *et al.*, 1989; Wood *et al.*, 1990), bacteriophage (Conkling *et al.*, 1976; Wood *et al.*, 1990), and lower eukaryotes (Malling and de Serres, 1973), where detailed molecular analysis had been undertaken. Moreover, it has been reported that A:T base pairs are more susceptible to radiation and oxidation-induced mutagenic damage than G:C pairs in a test strain (TA102) of *Salmonella* (Levin *et al.*, 1982). A similar sensitivity for A:T sites has been reported for ionizing radiation in *E. coli* (Basu *et al.*, 1989). These observations are consistent with the shift from G:C → A:T sites reported here.

In our study, 30% (27/90) of the deletions shared the common feature of not being readily explained by slippage events which may reflect the direct effects of ionizing

radiation-induced DNA strand breakage. This class of deletion was represented in our spectral sample at a frequency of approximately 2-fold greater than the frequency observed in the spontaneous database.

We further investigated the potential induction of microsatellite instability by exposure to ionizing radiation. We quantified the frequency of new alleles arising somatically in mononuclear cells of peripheral blood. Using fluorescent PCR and the ALF DNA sequencer (Pharmacia), combined with the ALF2SMA® analysis we demonstrated 5.3% and 4.5% of microsatellite alterations in non-exposed and exposed individuals, respectively. Our findings suggest that mismatch repair deficiency (or poor proficiency) is compatible with cell development. Our current approach lacked the sensitivity to discriminate between spontaneous and induced microsatellite instability and it is, therefore, not suitable for monitoring. On the other hand, our findings opened a window for the potential use of somatic microsatellite instability arising in normal population. This could become an effective tool in identifying individuals with somatic mutations in DNA repair genes, as well as in identifying clonal microsatellite alterations in body fluid which have important implications in cancer detection and diagnosis.

Finally, we examined the possible genetic risk imposed by radiation exposure in the Goiânia population. We examined both the genetic harm and the risk of carcinogenesis for that discrete population. We have attempted to use our experimental data on micronucleus and *hprt* mutant frequency to obtain some sense of the risk involved in human exposures. While this has provided a useful lesson, our small population size makes the interpretation of the results difficult. Interpretation is further complicated by the nature of the assumptions and the limited data upon which estimates are made. Nevertheless, our

estimates can be considered a reasonable indicator which is currently possible regarding the level of genetic damage for that population. We obtained risk estimates of about a 24-fold increase in dominant disorders in the first post-exposure generation of the directly exposed population. No detectable increase was found in the population at large. The risk of carcinogenesis in the directly exposed population was found to be increased by a factor in the range of 1.4 to 1.5. The added risk of carcinogenesis may be genuine and it is consistent with the number of individuals in the exposed population who had developed cancer by 1995. However, as it is rarely possible to categorically attribute a specific cancer to ionizing radiation, we can only conclude that it appears that the probability of carcinogenesis is indeed higher in the exposed population.

Keeping in mind that risk estimation encompasses complex calculations and is based upon a number of uncertainties and assumptions, we support Sobels' observations (Sobels, 1989 and 1990) that human somatic mutation could be used as a good indicator of ionizing radiation in humans. Therefore, extrapolations to the gametic level would be possible if the relationship between somatic and gametic mutations in mouse and humans were better understood. This would require the assumption that mice and humans are affected the same way which at this time, however, we cannot state unequivocally.

We attained the long term objective of this project for which we applied and developed methodologies for the purpose of monitoring a selected subset of individuals who unfortunately were exposed to ionizing radiation. Throughout the course of this work the patient population remained accessible and cooperative, contributing to helping future generations. They also silently and patiently hoped that we would bring some good news that would minimize their suffering and anxiety about the future and would reduce the

burden of being victims. Perhaps we bring good news at last. The general conclusions were that we have reported a decrease in mutant frequencies, a small risk of carcinogenesis, and an almost irrelevant risk for the next generation. The conclusions are all strongly supported by rigorous scientific work and literature. The results may even bring some degree of well-being back to the Goiânia exposed population, especially since no health consequences have yet been directly linked to radiation exposure. Post-accident traumatic stress and recurrences of primary radio-lesions have been the major causes of concern for the severely exposed individuals. Our conclusions should mostly reiterate the need for the continuation of long term follow up protocols, and clinical surveillance, with increased epidemiological support. Moreover, it should be emphasized that clinical follow-up and monitoring studies must be performed completely independently of socio-political programs.

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IX. Appendix

Appendix 1. Data from the micronucleus assay from cesium-137 exposed and non-exposed lymphocyte donors from Goiânia, Brazil. For group, 0 = control, 1 = direct exposure, 2 = focus 9, 3 = focus 2, 4 = staff. For the analyses, the values used for quantitative variables were sex (1 + male, 2 = female), smoking and alcohol use (0 = no, 1 = yes). A question mark represents an unknown value.

		Age				Dose	Total of	Micronucleus Distribution/Cell				
Obs	Group	(years)	Sex	Smoking	Alcohol	(rad)	Cells	One	Two	Three	Four	Five
1	0	15	1	1	0	0	1000	9	0	0	0	0
2	0	20	1	1	1	0	1000	7	0	0	0	0
3	0	31	2	1	1	0	1000	3	1	0	0	0
4	0	62	2	0	0	0	1000	3	1	0	0	0
5	0	28	1	1	1	0	1000	3	0	0	0	0
6	0	20	2	0	1	0	1100	2	1	0	0	0
7	0	59	1	1	1	0	1100	7	0	0	0	1
8	0	40	2	0	0	0	1000	2	1	1	0	0
9	0	24	2	0	0	0	1000	5	0	0	0	0
10	0	68	1	0	0	0	1000	5	1	0	0	0
11	0	30	2	0	1	0	1000	4	0	0	0	0
12	0	11	2	0	0	0	1000	2	1	0	0	0
13	0	17	1	0	0	0	1000	1	1	0	0	0
14	0	57	1	0	0	0	1000	4	0	1	0	0
15	0	39	2	1	0	0	1000	4	0	0	0	0
16	0	20	2	1	1	0	1000	9	0	0	0	0
17	0	30	2	1	1	0	1000	3	0	0	0	0
18	0	25	1	0	1	0	1000	6	1	0	0	0
19	0	53	1	0	0	0	1000	1	1	0	0	0
20	0	24	1	0	1	0	1000	2	1	0	0	0
21	0	39	2	1	1	0	1000	16	3	0	0	0
22	0	32	2	0	0	0	1000	3	0	0	0	0
23	0	16	2	0	1	0	1000	10	4	0	0	0
24	0	48	1	1	0	0	1000	20	5	0	0	0
25	0	31	2	1	0	0	1000	14	3	0	0	0
26	0	46	1	1	1	0	1000	12	3	0	0	0
27	0	30	2	1	0	0	1000	6	1	1	0	0
28	0	42	2	1	0	0	1000	6	0	0	0	0
29	0	40	2	1	1	0	1000	12	1	1	0	0
30	0	26	2	1	0	0	1000	8	2	1	0	0
31	0	6	1	0	0	0	1500	14	2	0	0	0
32	0	49	2	0	0	0	1200	7	0	0	0	0
33	0	20	2	0	1	0	1000	19	2	0	0	0
34	0	9	2	0	0	0	1400	6	1	0	0	0
35	0	7	2	0	0	0	1500	11	0	0	0	0
36	0	4	2	0	0	0	1500	8	0	1	1	0
37	0	76	2	0	0	0	1500	14	5	1	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell				
								One	Two	Three	Four	Five
38	0	25	2	0	1	0	1500	4	2	0	0	0
39	0	58	2	0	1	0	1500	6	2	0	0	0
40	0	40	2	1	1	0	1000	14	3	0	0	0
41	0	23	2	0	1	0	1000	5	0	0	0	0
42	0	53	1	0	1	0	1000	11	2	0	0	0
43	0	22	2	0	1	0	1500	18	1	0	0	0
44	0	24	2	0	0	0	1000	11	1	0	0	0
45	0	39	2	1	1	0	1500	13	4	0	0	0
46	0	50	2	0	1	0	1000	19	0	0	0	0
47	0	26	2	1	1	0	1500	6	0	0	0	0
48	0	25	2	0	1	0	1000	11	0	0	0	0
49	0	43	2	1	0	0	1500	21	1	0	0	0
50	0	15	1	0	0	0	1500	6	0	0	0	0
51	0	33	2	0	0	0	1000	10	1	0	0	0
52	0	18	2	0	0	0	1500	7	1	0	0	0
53	0	27	1	1	1	0	1500	15	0	0	1	0
54	0	43	2	0	0	0	1500	17	0	1	0	0
55	0	16	2	0	0	0	1500	6	0	0	0	0
56	0	24	1	1	1	0	1500	8	0	0	0	0
57	0	52	1	1	1	0	1500	19	1	0	0	0
58	0	34	2	0	0	0	1500	5	1	0	0	0
59	0	43	2	0	1	0	1500	10	1	0	0	0
60	0	60	1	1	0	0	1500	6	2	0	0	0
61	0	60	2	0	0	0	1500	21	2	0	0	0
62	0	26	2	1	0	0	1500	4	1	0	0	0
63	0	25	1	0	1	0	1500	8	2	1	0	0
64	0	29	2	0	0	0	1000	22	2	1	0	0
65	0	11	2	0	0	0	1000	4	0	0	0	0
66	0	46	2	0	0	0	1000	4	0	0	0	0
67	1	22	2	0	0	30	1500	19	5	0	0	0
68	1	21	1	0	0	620	1000	37	1	2	0	0
69	1	32	1	0	0	50	1000	28	1	0	0	0
70	1	42	2	1	1	60	1000	11	3	0	0	0
71	1	27	2	1	1	45	1000	19	1	0	0	0
72	1	62	2	0	0	40	1000	21	1	0	0	0
73	1	65	1	1	0	20	1200	5	0	0	0	0
74	1	30	2	1	1	15	1000	15	1	0	0	0
75	1	43	2	1	1	120	1000	27	5	1	0	0
76	1	46	2	1	1	30	1000	26	2	0	0	0
77	1	60	2	1	1	0	1000	31	1	0	0	0
78	1	26	1	1	0	15	1000	7	2	0	0	0
79	1	14	1	0	0	130	1500	12	0	0	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell One	Two	Three	Four	Five
80	1	37	2	0	0	20	1500	9	3	0	0	0
81	1	38	1	1	1	700	1000	37	7	2	0	0
82	1	28	2	0	0	110	1500	10	0	0	0	0
83	1	29	2	0	0	100	1000	16	2	0	0	0
84	1	47	1	1	1	210	1500	7	1	0	0	0
85	1	41	1	1	0	300	1000	14	2	0	0	0
86	1	42	1	1	1	440	1000	21	1	1	0	0
87	1	32	1	1	1	100	2000	14	3	0	0	0
88	1	58	2	0	0	430	1000	34	4	0	0	0
89	1	21	1	0	0	270	1000	20	1	0	0	0
90	1	13	1	1	0	290	1000	18	0	0	0	0
91	1	22	1	1	1	290	1500	19	2	0	0	0
92	1	51	1	1	0	50	1000	5	1	0	0	0
93	1	13	1	1	1	160	1000	18	2	0	0	0
94	1	7	2	0	0	20	1000	16	0	0	0	0
95	1	28	2	0	0	40	1000	11	2	0	0	0
96	1	9	2	0	0	30	1000	9	3	0	0	0
97	1	6	2	0	0	70	1000	16	0	1	1	0
98	1	39	2	1	1	15	1000	20	1	0	0	0
99	1	8	1	0	0	50	1000	8	0	0	0	0
100	1	31	1	1	0	30	1000	4	1	0	0	0
101	1	15	1	1	0	50	1000	6	0	0	0	0
102	1	2	2	0	0	20	1000	10	0	0	0	0
103	1	29	2	0	0	20	1000	15	0	0	0	0
104	1	30	1	1	1	20	1500	6	1	0	0	0
105	1	18	1	1	1	10	1000	7	0	0	0	0
106	1	3	2	0	0	0	1000	5	1	0	0	0
107	1	29	2	0	0	40	1500	6	0	0	0	0
108	1	37	2	0	0	30	1000	6	0	0	0	0
109	1	5	1	0	0	0	1500	11	0	0	0	0
110	1	7	2	0	0	20	1500	8	1	1	0	0
111	1	7	2	0	0	10	1500	6	1	0	0	0
112	1	9	2	0	0	40	1000	5	0	0	0	0
113	1	6	1	0	0	10	1000	7	0	0	0	0
114	1	11	1	0	0	20	1000	9	0	0	0	0
115	1	7	1	0	0	60	1000	13	0	0	0	0
116	1	13	1	0	0	30	1000	22	2	0	0	0
117	1	34	2	1	1	20	1000	5	0	0	0	0
118	1	7	1	0	0	20	1000	7	2	0	0	0
119	1	6	2	0	0	20	1000	4	0	0	0	0
120	1	7	1	0	0	40	1000	4	0	0	0	0
121	1	27	2	0	0	30	1000	10	0	0	0	0
122	1	50	2	0	0	10	1500	25	3	0	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell One	Two	Three	Four	Five
123	1	22	1	0	0	20	1000	16	2	0	0	0
124	1	26	2	0	0	20	1000	7	1	0	0	0
125	1	45	1	0	0	50	1000	12	3	0	0	0
126	1	16	2	1	1	50	1000	14	1	1	0	0
127	1	6	1	0	0	20	1000	7	0	0	0	0
128	1	28	1	0	0	10	1000	15	1	0	0	0
129	1	24	1	1	1	30	1000	15	4	1	0	0
130	1	26	1	1	1	20	1000	18	4	1	0	0
131	1	2	1	0	0	0	1000	8	0	0	0	0
132	1	23	2	0	0	40	1000	14	0	0	0	0
133	1	1	2	0	0	0	1000	6	1	0	0	0
134	1	33	2	0	0	10	1000	13	0	1	0	0
135	1	9	1	0	0	25	1000	14	0	0	0	0
136	1	7	1	0	0	0	1000	6	0	0	0	0
137	1	14	2	0	0	40	1000	9	1	0	0	0
138	1	9	2	0	0	15	1000	15	0	0	0	0
139	1	31	2	1	1	20	1000	8	1	0	0	0
140	1	2	2	0	0	15	1000	7	0	0	0	0
141	1	36	1	0	0	20	1000	11	1	0	0	0
142	2	27	2	1	0	?	1000	3	0	0	0	0
143	2	25	2	1	1	?	1000	14	0	0	0	0
144	2	35	1	1	1	?	1000	8	1	0	0	0
145	2	60	2	1	0	?	1000	4	0	0	0	0
146	2	59	2	0	1	?	1000	10	0	0	0	0
147	2	16	2	0	1	?	1000	4	0	0	0	0
148	2	35	1	1	1	?	1000	6	1	0	0	0
149	2	60	2	1	0	?	1000	4	1	0	0	1
150	2	54	2	0	1	?	1000	4	0	0	0	0
151	2	30	1	1	1	?	1000	5	0	0	0	0
152	2	52	2	1	0	?	1000	5	0	0	0	0
153	2	37	2	0	0	?	1000	7	0	0	0	0
154	2	22	2	0	0	?	1000	4	0	0	0	0
155	2	38	2	0	0	?	1000	7	0	0	0	0
156	2	18	1	0	0	?	1000	9	2	0	0	0
157	2	20	2	0	0	?	1000	6	0	0	0	0
158	2	19	2	0	1	?	1000	9	0	0	0	0
159	2	17	1	0	1	?	1000	16	0	0	0	0
160	2	58	1	1	1	?	1000	3	0	0	0	0
161	2	30	2	1	1	?	1000	2	1	0	0	0
162	2	43	1	1	0	?	1000	4	0	0	0	0
163	2	38	1	1	1	?	1000	7	0	0	0	0
164	2	26	2	0	0	?	1000	8	1	1	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell One	Two	Three	Four	Five
165	2	20	2	0	0	?	1000	11	2	0	0	0
166	2	67	1	1	0	?	1000	11	0	0	0	0
167	2	29	1	0	1	?	1000	4	0	0	0	0
168	2	46	1	1	1	?	1000	5	0	0	0	0
169	2	35	1	1	1	?	1000	6	1	0	0	0
170	2	18	2	0	1	?	1000	5	0	0	0	0
171	2	39	2	1	1	?	1000	9	0	0	0	0
172	2	63	1	0	0	?	1000	5	0	0	0	0
173	2	39	2	0	0	?	1000	7	0	0	0	0
174	2	17	1	0	0	?	1000	9	1	0	0	0
175	2	59	2	0	0	?	1000	14	0	0	0	0
176	2	22	1	0	1	?	1000	3	0	0	0	0
177	2	67	2	0	0	?	1000	14	0	0	0	0
178	2	33	2	0	0	?	1000	9	1	0	0	0
179	2	72	2	1	0	?	1000	22	0	0	0	0
180	2	44	1	1	1	?	1000	6	1	0	0	0
181	2	54	2	0	0	?	1000	11	1	0	0	0
182	2	21	2	0	1	?	1000	3	1	0	0	0
183	2	24	2	0	0	?	1000	9	1	0	0	0
184	2	27	2	0	0	?	1000	3	0	0	0	0
185	2	71	2	0	0	?	1000	13	1	1	0	0
186	2	41	2	0	0	?	1000	11	1	0	0	0
187	2	40	2	1	0	?	1000	17	0	0	0	0
188	2	51	1	1	1	?	1000	16	1	0	0	0
189	2	15	1	0	0	?	1000	5	0	0	0	0
190	2	9	2	0	0	?	1000	5	0	0	0	0
191	2	73	2	0	0	?	1000	9	1	0	0	0
192	3	51	2	0	0	?	1000	19	1	0	0	0
193	3	52	2	0	0	?	1000	12	1	0	0	0
194	3	56	2	0	0	?	1000	14	1	0	0	0
195	3	54	2	0	0	?	1000	10	0	0	0	0
196	3	75	2	0	0	?	1000	7	0	0	0	0
197	3	38	2	0	0	?	1000	11	1	1	0	0
198	3	62	2	0	0	?	1200	11	1	0	0	0
199	3	14	1	0	0	?	1000	8	1	0	0	0
200	3	50	2	0	0	?	1200	16	2	0	0	0
201	3	14	2	0	0	?	1000	11	1	0	0	0
202	3	12	1	0	0	?	1000	10	0	0	0	0
203	3	28	2	0	0	?	1000	8	0	0	0	0
204	3	27	2	0	0	?	1000	8	1	0	1	0
205	3	50	2	1	0	?	1000	5	2	0	0	0
206	3	29	2	0	0	?	1000	8	1	0	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell				
								One	Two	Three	Four	Five
207	3	31	2	0	0	?	1000	4	1	0	0	0
208	3	36	2	0	0	?	1000	11	0	0	0	0
209	3	60	1	0	0	?	1000	9	0	0	0	0
210	3	53	2	0	0	?	1000	11	1	0	0	0
211	3	25	2	0	0	?	1000	13	0	0	0	0
212	3	20	1	0	0	?	1000	6	1	0	0	0
213	3	41	2	0	0	?	1000	7	0	0	0	0
214	3	26	1	0	0	?	1000	9	1	0	0	0
215	3	33	1	0	0	?	1000	10	2	0	0	0
216	3	33	2	0	0	?	1000	11	2	0	0	0
217	3	20	1	0	0	?	1000	6	2	0	0	0
218	3	16	1	0	0	?	1500	10	0	0	0	0
219	3	21	2	0	0	?	1000	4	1	0	0	0
220	3	25	2	0	0	?	1000	7	0	0	0	0
221	3	44	2	0	0	?	1000	12	0	0	0	0
222	3	60	2	0	0	?	1000	12	1	0	0	0
223	3	50	2	0	0	?	1000	6	0	1	0	0
224	3	33	1	0	0	?	1100	9	0	0	0	0
225	3	20	2	0	0	?	1500	6	1	0	0	0
226	3	42	2	0	0	?	1000	7	1	0	0	0
227	3	40	2	0	0	?	1000	8	0	0	0	0
228	3	40	2	0	0	?	1000	10	0	0	0	0
229	3	30	2	0	0	?	1000	9	1	0	0	0
230	3	29	1	0	0	?	1000	16	0	0	0	0
231	3	27	1	0	0	?	1000	9	0	0	0	0
232	3	54	1	0	0	?	1000	11	2	0	0	0
233	3	52	1	0	0	?	1000	8	0	0	0	0
234	3	33	2	0	0	?	1000	8	0	0	0	0
235	3	33	2	0	0	?	1000	9	0	0	0	0
236	3	57	2	0	0	?	1000	16	3	0	0	0
237	3	39	2	0	0	?	1000	10	1	0	0	0
238	3	50	1	0	0	?	1000	5	1	0	0	0
239	3	42	1	0	0	?	1000	6	2	0	0	0
240	3	31	1	0	0	?	1000	10	1	0	0	0
241	3	26	2	0	0	?	1000	11	0	0	0	0
242	3	50	1	0	0	?	1000	12	2	0	0	0
243	3	50	1	0	0	?	1000	9	0	0	0	0
244	3	46	2	0	0	?	1000	10	0	0	0	0
245	3	37	1	0	0	?	1000	13	2	0	0	0
246	3	49	2	0	0	?	1000	7	0	0	0	0
247	3	47	1	0	0	?	1000	10	10	0	0	0
248	3	60	2	0	0	?	1000	11	1	0	0	0

Obs	Group	Age (years)	Sex	Smoking	Alcohol	Dose (rad)	Total of Cells	Micronucleus Distribution/Cell One	Two	Three	Four	Five
249	3	47	1	0	0	?	1000	13	0	0	0	0
250	3	49	2	0	0	?	1000	6	2	0	0	0
251	3	16	2	0	0	?	1000	7	0	0	0	0
252	3	15	1	0	0	?	1000	9	1	0	0	0
253	3	25	1	0	0	?	1000	7	2	0	0	0
254	3	24	1	0	0	?	1000	13	1	0	0	0
255	3	11	1	0	0	?	1000	20	0	0	0	0
256	3	36	2	0	0	?	1000	11	2	0	0	0
257	3	50	2	0	0	?	1000	12	1	0	0	0
258	3	35	2	0	0	?	1000	10	0	0	0	0
259	4	25	2	0	0	0	1000	15	1	0	0	0
260	4	40	2	0	0	0	1000	18	3	0	0	0
261	4	43	2	0	0	0	1000	12	1	0	0	0
262	4	32	2	0	0	0	1000	13	3	0	0	0
263	4	40	2	0	0	0	1000	19	1	1	0	0
264	4	41	2	0	0	0	1000	12	2	1	0	0
265	4	56	1	0	0	0	1000	15	1	0	0	0
266	4	54	1	0	0	0	1000	15	0	0	0	0
267	4	46	1	0	0	15	1000	11	1	0	0	0
268	4	46	2	0	0	0	1000	15	2	0	0	0
269	4	31	2	0	0	20	1000	19	2	0	0	0
270	4	31	2	0	0	0	1000	17	2	0	0	0
271	4	27	1	0	0	15	1000	15	1	0	0	0
272	4	51	1	0	0	30	1000	17	0	0	0	0
273	4	32	2	0	0	0	1000	11	2	0	0	0
274	4	44	1	0	0	0	1000	11	1	0	0	0
275	4	25	1	0	0	0	1000	19	0	0	0	0
276	4	30	2	0	0	0	1000	14	0	0	0	0

Appendix 2a. Definition of units of measurement of radiation energy.

Unit of Radiation	Concept	Definition	Former Unit	Equivalency
Becquerel (Bq)	Unit of activity	The quantity of radioactive material in which one atom is transformed per second	Curie (Ci) [activity of 1g ^{226}Ra] [3.7×10^{10} dps]	1 Bq = 27 pCi = 1 dps
Coulomb/Kilogram (C/Kg)	Unit of radiation exposure	The measure of ionization produced in air by X- and γ-rays	Roentgen (R)	1 R = 2.58×10^{-4} C/Kg
Gray (Gy)	Unit of absorbed dose	The mean energy imparted to matter by ionizing radiation per unit mass of irradiated material at a point of interest (Joules/Kg)	rad	1Gy = 1 J/Kg = 100 rad
Sievert (Sv)*	Unit of relative biological effectiveness (RBE)	The product of the absorbed dose (Gy) by the effective quality factor (Q) [see appendix 2b]	rem	1 Sv = 100 rems
Effective Dose Equivalent (H_E) (Sv)	Unit of damage	The sum of the weighted H_B for irradiated tissues or organs (see appendix 2c) [$H_B = \sum_T w_T H_T = H_{wb}$]	NA	NA
Committed Dose Equivalent (H_{50}) [Sv]	Unit of damage	The time integral of the dose equivalent rate in a specific tissue following radionucleide intake	NA	NA

* 1 Sv = 1 Gy, in that the RBE equal the absorbed dose.

Appendix 2b. Recommended values of effective quality factor for different types of radiation.

Type of Radiation	Approximate value of Q
X-rays, γ-rays, β particles, and electrons	1
Thermal neutrons	5
Neutrons, protons, α particles, and multi-charged particles of unknown energy	20

Appendix 2c. Recommended values of weighting factors (w_T) for calculating effective dose equivalent.

Tissue	Risk Coefficient	w_T
Gonads	40×10^{-4}/Sv	0.25
Breast	25×10^{-4}/Sv	0.15
Red bone marrow and lung	20×10^{-4}/Sv	0.12
Thyroid and bone surfaces	5×10^{-4}/Sv	0.03
Remainder	50×10^{-4}/Sv	0.30